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ENDOCRINOLOGY

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TREATMENT OF SUCCESSIVE GENERATIONS OF RATS WITH THYMUS EXTRACT (HANSON) AND RELATED SUBSTANCES[1]

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DETROIT, MICHIGAN

IN 1930 HANSON (1) prepared a thymus extract which he hoped would prove a cure for certain types of cancer. Large quantities of this extract were turned over to Rowntree for biological study at The Philadelphia Institute for Medical Research. In 1934 Rowntree, Clark, Steinberg and Hanson (2) reported that the injection of this extract into successive generations of Wistar rats induced a remarkable precocity of growth and development which was noticeable in the 3rd generation and which became more marked in each of the five generations studied. In a more recent paper (3) they report 12 generations under study in which each generation showed a still greater precocity than the preceding generation.

In searching for the active principle it was found (3) that all biologically potent extracts contained at least 15 mg.% of iodine reducing substances (calculated as gluthathione after the method of Woodward and Fry, 4). More recently in 1938 (5) the group has stated "It has also been found that all biologically potent extracts of thymus contained appreciable amounts of iodine-reducing material (50 to 258 mg. % calculated as glutathione) by the method of Woodward and Fry and that this was low or absent in all biologically inactive preparations." The Philadelphia group has reported further (6) that this iodine-reducing titer can be accounted for as glutathione, cysteine and ascorbic acid, and that the injection of either gluthathione alone or in combination with ascorbic acid induced a pattern of response similar to that observed in animals treated with thymus extract. In the discussion at the presentation of this latter paper Lee stated that he had been able to produce noticeable precocity in the 4th generation of Wistar rats which had been treated continuously with glutathione throughout each generation.

In the studies of Rowntree and his co-workers the solutions of glutathione and ascorbic acid were prepared immediately before injection, while in Lee's study a stock solution was prepared each week and dilutions were made daily. Lee used 0.5 mg. to 1 mg. of glutathione daily while the Philadelphia group used from 0.25 mg. to 2 mg. daily with optimal results at 2 mg.

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[1] This study was aided by a grant from the Committee on Scientific Research of the American Medical Association and Works Progress Administration Project No. 665-51-3-225.

To our knowledge there have been no reports confirming the finding of a precocity after treatment of successive generations of rats with thymus extract (Hanson). On the other hand there have been several unsuccessful attempts at reproducing this phenomenon.

Einhorn and Rowntree have reported that thymectomy through successive generations produces an accruing retardation. These results have lacked confirmation in other laboratories (7, 8).

MATERIALS AND METHODS

In all, from Feb. 15, 1938 to Nov. 3, 1939, forty-two separate batches of extract were received from Hanson[2] for injection. This is approximately one batch of extract every 2 weeks. The majority of the shipments were made in refrigerated containers. All except the first 7 were analyzed for iodine-reducing content by the method of Woodward and Fry (4). Only 4 batches showed an iodine-reducing titer of less than 80 mg.% at the time of arrival in Detroit, the lowest of these being 55 mg.%. These lots with a low iodine-reducing titer were replaced as rapidly as possible with more potent extracts. The majority of the extracts showed a titer of more than 100 mg.%, in fact, of the last 14 batches of extract only one (96 mg.%) was below 100 mg.%. The average iodine-reducing titer for all extracts at receipt in our laboratory was 100 mg.% with a range from 55 mg.% to 149 mg.%.

The animals used as controls and for treatment with thymus extract (Hanson), glutathione or glutathione-ascorbic acid[3] were adult albino rats taken from our stock colony. These animals were fed our stock diet of Ti-O-Ga dog chow and water with the semi-weekly addition of lettuce. Another group of animals for treatment with thymus extract (Hanson) was obtained from the experimental colony at the Wistar Institute. These animals were maintained on the diet used at the Philadelphia Institute for Medical Research, but prepared in our laboratory. The last group of animals were of the Wistar strain of rats maintained at the Philadelphia Institute for Medical Research and sent to us through the kindness of Doctor Rowntree and Doctor Einhorn.[4] These latter animals were fed the Institute diet which was prepared in Philadelphia and shipped as needed to Detroit.

The glutathione and glutathione-ascorbic acid for injection in our strain of rats were prepared as solutions in normal saline. The solutions represented 1 mg. of glutathione per cc. and 1.5 mg. of ascorbic acid per cc. and were prepared fresh twice weekly and kept in the refrigerator. At Dr. Rowntree's suggestion the Wistar rats obtained from the Philadelphia Institute for Medical Research were injected with a solution of 2 mg. of glutathione and 10 mg. of ascorbic acid per cc. This last solution was prepared immediately before injection each day. All of the injected animals received 0.25 cc. when they reached 25 gm., 0.50 cc. at 50 gm., 0.75 cc. at 75 gm. and 1 cc. at 100 gm. and thereafter.

As we have previously stated, all of the animals were weighed daily until weaning at 25 days and at weekly intervals thereafter. All animals which were not intended

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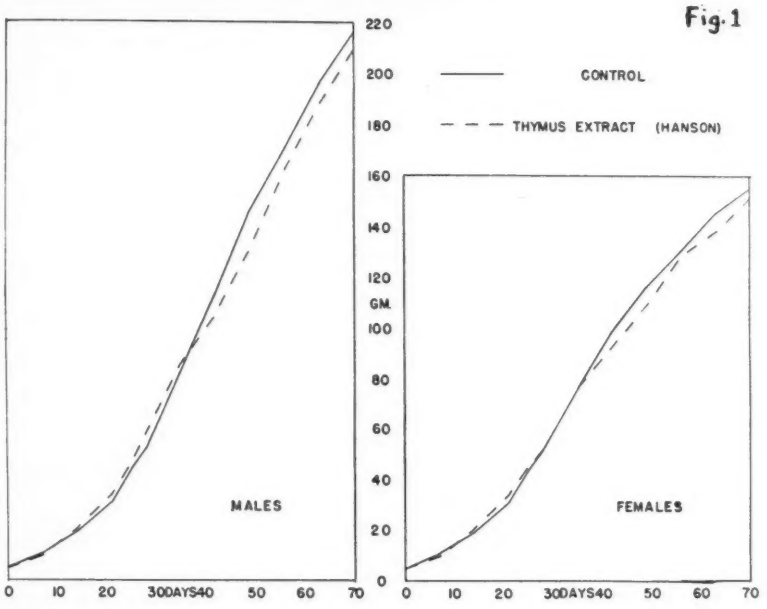
[2] We would like to take this opportunity of thanking Doctor Adolph M. Hanson and his assistant Mr. George W. Hanson, Faribault, Minnesota, for their generosity and unstinting labor in maintaining a regular and constant supply of the thymus extract.

[3] The glutathione used in these studies was purchased either from Hoffman-LaRoche, Nutley, N. J. or from the Pfansteil Company, Waukegan, Ill. The ascorbic acid was supplied by Parke, Davis & Company, Detroit, Mich., through the courtesy of Doctor Oliver Kamm and Doctor D. A. McGinty.

[4] We are greatly indebted to Doctor L. G. Rowntree and Doctor N. H. Einhorn of the Philadelphia Institute for Medical Research for their generosity in supplying these animals and the necessary diet. Doctor Einhorn also made his original animal records available for our use. We are indebted also to Doctor Rowntree and Doctor Einhorn for many courtesies extended to one of us (A. S.) during a period of several days spent in their laboratory observing methods and technics.



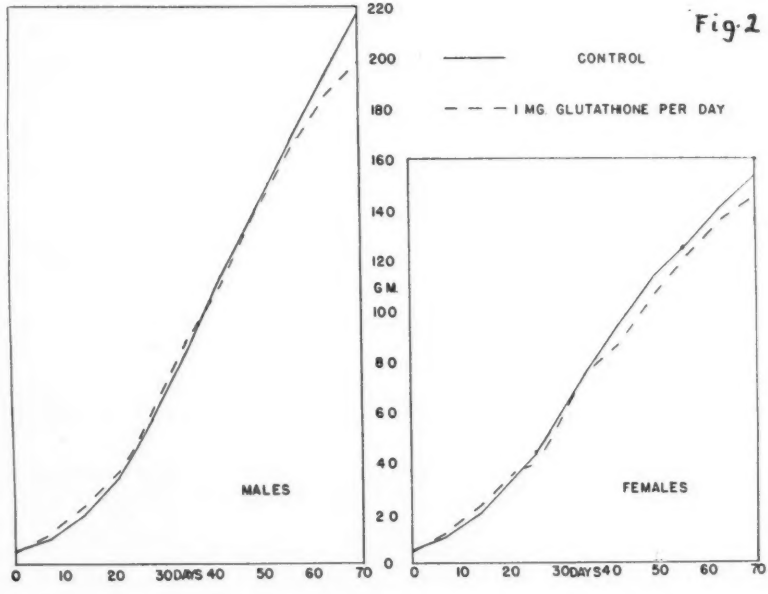


Fig. 1. Growth curves of 6th generation normal rats and 6th generation rats injected with thymus extract (Hanson). Fig. 2. Growth curves of the 4th and 5th generations of normal rats and rats injected with glutathione.

TABLE 1. DEVELOPMENT OF NORMAL RATS AND RATS INJECTED WITH THYMUS EXTRACT (HANSON)

Series	Vaginal canalization			1st estrus	Testes descend	Ears open	Incisors erupt	Eyes open
	Weight	Body length	Age					
	gm.	*mm.*	*days*	*days*	*days*	*days*	*days*	*days*
6th generation								
Normal	85±16.2	137±10.9	40±5.3	40±5.5	19±1.4	2.5	11.0	15.8
Treated	102± 9.5	150± 6.7	46±5.9	47±5.0	18±0.4	3.4	10.4	15.3
5th generation								
Normal	81± 9.3	138± 4.4	36±2.8	37±7.8	19±0.9	2.5	10.3	15.7
Treated	91± 9.7	142± 7.3	37±3.0	38±3.5	19±1.2	2.6	10.5	15.4
4th generation								
Normal	85±16.2	137±10.9	40±5.3	40±5.5	19±1.4	2.8	11.1	16.4
Treated	95±16.2	148± 9.2	42±5.7	44±7.4	19±2.3	3.4	10.0	15.5

TABLE 2. BODY WEIGHTS AND DIMENSIONS OF 70 DAY OLD NORMAL RATS AND RATS INJECTED WITH THYMUS EXTRACT (HANSON)

	Animals	Body weight	Body length	Tail length	Length forelimbs	Length hind limbs
	No.	*gm.*	*mm.*	*mm.*	*mm.*	*mm.*
			Males			
6th generation						
Normal	25	216±21	195±6	165± 9	160±4	210± 6
Treated	9	209±17	192±4	165± 6	163±3	210± 4
5th generation						
Normal	27	223±18	197±6	171±10	165±5	213± 5
Treated	19	237±24	199±7	181±10	164±3	217± 5
4th generation						
Normal	31	218±25	201±7	169± 8	162±5	210± 6
Treated	19	233±26	201±6	180± 7	165±8	214± 7
			Females			
6th generation						
Normal	23	155±13	179±7	154± 7	154±6	195±13
Treated	11	151± 6	177±4	157± 4	153±2	198± 3
5th generation						
Normal	31	157±12	178±5	160± 7	154±4	196± 4
Treated	22	174±15	186±5	174± 8	157±8	202± 4
4th generation						
Normal	31	153±12	179±4	156± 8	153±3	194± 4
Treated	20	167±15	186±4	160± 6	155±5	199± 3

for use in carrying on the next generation were autopsied at 70 days. Controls were housed in adjacent cages and handled daily. Injections and observations were made at the same time each day.

The autopsy procedure and other methods of care and measurement of the animals were identical with those which we have employed in all of our studies on the thymus and which has been reported elsewhere (9).

RESULTS

The data obtained during growth and at autopsy are presented with their standard deviations. This includes data obtained for vaginal canalization and testicular de-

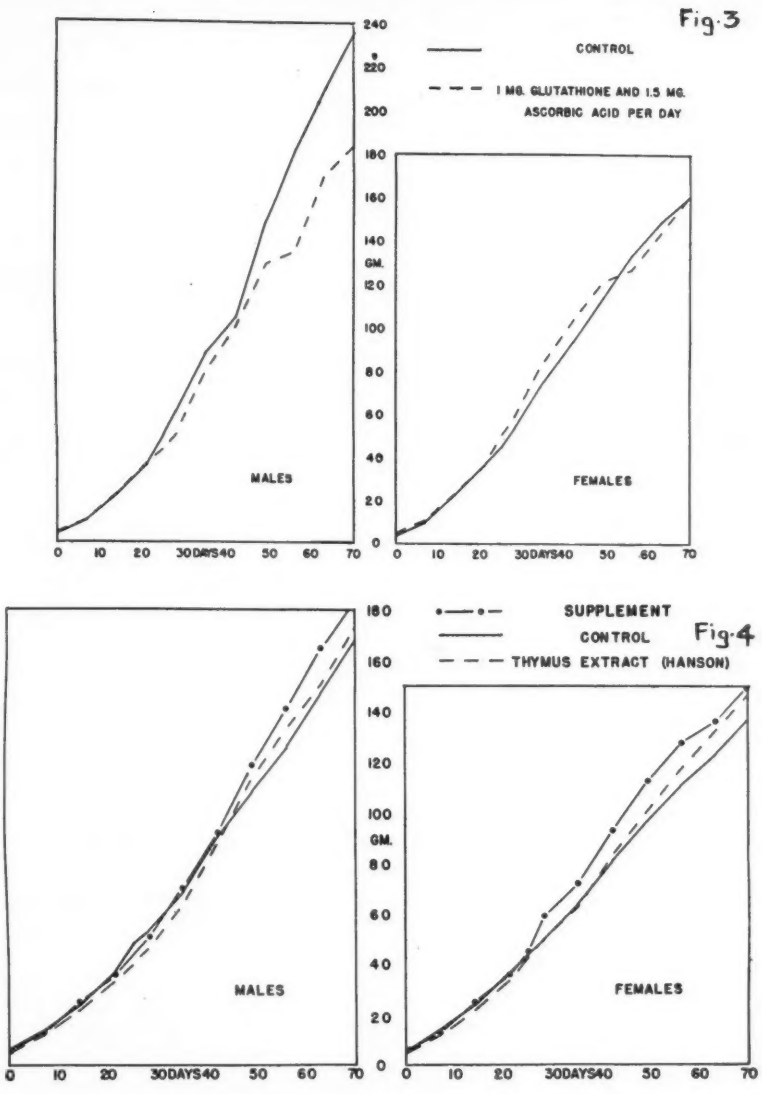


Fig. 3. Growth curves of the 3rd and 4th generations of normal rats and rats injected with glutathione and ascorbic acid. Fig. 4. Growth of Wistar rats on the Rowntree diet.

scent. The data for ear openings, tooth eruptions and eye openings are presented as the 50% points on curves plotted on a percentage-day basis.[5,6]

[5] In the interest of space conservation the tables include data for the later generations only.

[6] The standard deviations of the means were calculated by the formula $s = \sqrt{\frac{Sx^2}{(n-1)}}$ where s = standard deviation, S = sum, x = deviation from the mean, and n = number of units as suggested by G. W. Snedecor, Statistical Methods. Collegiate Press, Ames, Iowa, 1938.

TABLE 3. ORGAN WEIGHTS OF 70 DAY OLD NORMAL RATS AND RATS INJECTED WITH THYMUS EXTRACT (HANSON)

	Thymus	Kidney	Adrenals	Thyroid	Pituitary	Heart	Testis	Seminal Vesicle	
								Full	Empty
	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
Males									
6th generation									
Normal	477±68	1,050±156	35± 5	18±4	8.4±1.2	785± 89	1,235±146	298±60	136±40
Treated	349±46	1,051±114	31± 7	17±6	7.5±1.9	809± 74	938±264	196±56	105±17
5th generation									
Normal	426±94	1,023±128	37± 7	18±3	8.7±2.0	793± 23	1,276±116	312±89	162±28
Treated	350±58	1,116±183	40± 8	19±5	9.0±1.2	845±108	1,020±437	317±89	158±38
4th generation									
Normal	356±47	1,063±119	39± 8	17±5	8.0±1.4	791± 77	1,229±108	345±55	166±25
Treated	439±67	1,108±142	42± 9	20±7	7.8±1.6	825± 91	1,170±104	245±64	125±41
Females							Ovaries		
6th generation									
Normal	378±69	787± 98	45±10	17±3	9.8±1.8	612±58	59± 15		
Treated	282±37	855±103	48±10	17±4	8.8±1.8	632±51	46± 10		
5th generation									
Normal	287±39	724± 77	39± 4	15±2	8.0±1.3	576±64	54± 7		
Treated	294±55	809± 66	55±10	18±3	9.5±1.0	627±71	52± 6		
4th generation									
Normal	311±39	728± 75	46± 9	17±5	8.5±2.9	596±41	55± 12		
Treated	353±47	803± 70	45± 8	18±4	7.4±1.4	638±52	51± 10		

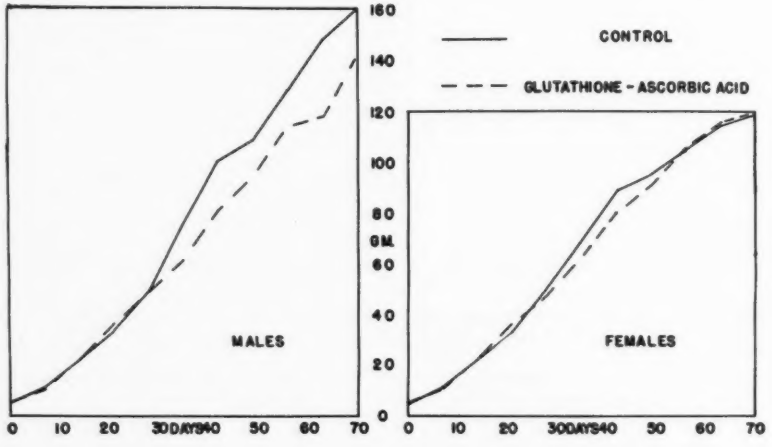


Fig. 5. GROWTH CURVES OF 3RD, 4TH AND 5TH GENERATIONS OF ROWNTREE RATS ON ROWNTREE DIET.

TABLE 4. DEVELOPMENT OF RATS UNDER EXPERIMENTAL CONDITIONS

Series	No.	Vaginal canalization			1st estrus	Testes descend	Ears open	Incisors erupt	Eyes open
		Weight	Body length	Age					
		gm.	mm.	days	days	days	days	days	days
4th and 5th generation									
Normal	58	81± 9.3	138± 4.4	36± 2.8	37± 2.8	19±0.9	2.5	10.3	15.7
Glutathione	22	107±10.8	155± 4.8	50± 7.0	51± 7.9	19±0.5	2.7	10.8	15.5
3rd and 4th generation									
Normal	62	78± 9.9	137± 5.1	36± 2.7	38± 2.8	18±1.6	2.8	11.1	16.4
Glutathione-ascorbic acid	16	90±20.6	144±10.7	38± 3.3	40± 4.7	19±0.2	3.3	11.1	16.0
Wistar rats on Rowntree diet									
Normal	31	95± 8.9	148± 5.4	51± 7.2	51± 9.3	19±1.0	2.2	8.8	14.1
Extract (Hanson)	39	103±10.7	151± 7.1	50± 5.6	52± 5.5	20±1.4	2.5	8.8	14.7
Supplement[1]	32	86± 6.5	141± 7.1	40± 2.6	40± 2.7	19±1.7	2.0	8.8	13.9
3rd, 4th and 5th generations of Rowntree rats on Rowntree diet									
Normal	25	99±13.8	151±12.4	51±11.7	52±11.7	18±1.7	2.5	8.3	14.8
Glutathione-ascorbic acid	21	96±17.0	152± 4.7	63±16.1	65±16.2	24±4.1	2.7	9.5	15.1

[1] 3% dried brewer's yeast and 3% wheat germ added to diet.

Examination of figure 1 shows that thymus extract (Hanson) injected intraperitoneally had no effect on the growth of males or females. In table 1, which shows the developmental data on the same animals, it is apparent that there was no acceleration in any of the developmental criteria which we have been able to employ. The autopsy data in tables 2 and 3 show no significant differences between experimental and control animals.

The 4th and 5th generations of our normal animals injected with 1 mg. of glutathione daily are presented in figure 2 and table 4. With regard to vaginal canalization in the females of this group, there appeared to be a slight delay in the injected animals. Figure 3 and table 4 contain data on normal animals injected daily with a combination of glutathione and ascorbic acid. Again the injected animals were very slightly delayed. Figure 4 and table 4 summarize data on the rats obtained from Wistar Institute fed on the Rowntree diet. Those labeled "supplement" were fed 3% dried brewer's yeast and 3% wheat germ in addition to the regular diet. The observations on this group have suggested the possibility that the diet employed by the Philadelphia Institute for Medical Research has certain deficiencies and is inadequate for normal development. This view is further substantiated by a comparison of these animals with our own colony controls. The final group of animals are those obtained from the Philadelphia Institute for Medical Research and fed during the course of the experiment on

their diet as supplied by them. The summaries for the 3rd, 4th and 5th generations are presented in figure 5 and table 4 and show conclusively that treatment with glutathione and ascorbic acid failed to exert any accelerative influences. The difference in age of vaginal canalization and of first estrus between normal and injected animals is partially explained by the fact that 3 animals of the control group were autopsied at 70 days at which time their vaginae were not canalized. These 3 animals were omitted in the calculation of the figures given.

DISCUSSION

Allardyce et al. (10) failed to accelerate development in 4 generations of rats injected with thymus extract (Hanson). These extracts were reported as having high titers of iodine-reducing substances.

Nelson and McGinty (11) were unable to advance the rate of growth or development although they used parts of the original batches of thymus extract (Hanson) which were sent to Doctor Rowntree.

Chiodi (12) reported failure to accelerate growth or maturity in 5 generations of rats which had been injected with thymus extract (Hanson).

Vickery (13) using thymus extract (Hanson) supplied to him by Doctor Rowntree was unable to detect any evidence of precocity in an unstated number of generations.

Smith and Jones (14) reported that 3 generations of strain A mice showed no evidence of unusual growth or development. Lafon (15) observed no significant acceleration in growth, but mentioned a slight precocity of sexual development.

We have reported in an earlier paper (8) that thymectomy in 6 successive generations failed to influence growth and development. These results are in agreement with the report of Chiodi (7) who found no effect from 5 generations of thymectomies. Furthermore, we have been unable to effect an acceleration in growth or development in 6 successive generations of animals which were injected with thymocrescin prepared by Asher's method (9).

In the work which is being reported at this time we have been particularly fortunate in having the full cooperation of Doctor Hanson, Doctor Einhorn and Doctor Rowntree. Extracts, animals, dietary materials and advice have been placed at our disposal in the attempt we have made to duplicate as closely as possible the conditions of Doctor Rowntree's original experiment. Efforts were made to employ highly standardized methods throughout every phase of the various experiments and to evaluate our data carefully. To this end we have considered animals of each sex separately by means of growth curves and statistical analyses. The result has been a study which would appear to be the most thorough and comprehensive attempt that has been made to confirm the results of Rowntree and his co-workers.

Although we have failed to obtain any evidence of a specific relation between the thymus and accelerated growth or maturation, some of the results have offered a partial explanation for the phenomenon which was observed by Rowntree's group. It seems possible that dietary deficiencies may have played a very important role in their studies. Inferior growth and delayed maturation of control animals would provide a striking basis for comparison with any corrective influences which might be exerted by the thymic extract. Attention is directed to the group of Wistar rats which were fed the Rowntree diet supplemented with yeast and wheat germ. Although these animals showed relatively little acceleration of growth, the data for vaginal canalization exhibited a decided shift. A study of the data in table 4 will emphasize this point. It is apparent that the delay in age and size of the animals at vaginal canalization, which is so apparent in the Wistar animals fed on the unsupplemented diet, was corrected by supplementary feeding and the figures for that event were brought within the range

shown by animals in our colony. It is important to note that the acceleration was produced in the first litter of the original animals placed on this supplemented diet and is of about the magnitude of the acceleration reported by the Rowntree group to follow every treatment with the exception of thymus extract (Hanson). This view is supported by Vickery's statement(13) that all of his animals which were fed on the diet used by the Philadelphia Institute for Medical Research were smaller at all ages than those fed on the diet used by the Connecticut Agricultural Experimental Station.

SUMMARY

We have been unable to induce any acceleration of growth or development in the following groups of animals: (*a*) rats from our albino colony treated for 6 generations with preparations of thymus extract prepared by Doctor Hanson; (*b*) rats from our albino colony treated for 5 generations with 1 mg. per day of crystalline glutathione; (*c*) rats from our albino colony treated for 4 generations with 1 mg. of crystalline glutathione plus 1.5 mg. of crystalline ascorbic acid; (*d*) rats obtained from the experimental colony of the Wistar Institute, fed a diet recommended by the Philadelphia Institute for Medical Research and prepared in our laboratory, and injected with preparations of thymus extract (Hanson) for 4 generations; (*e*) rats of the Wistar strain obtained from the Philadelphia Institute for Medical Research, fed their diet as supplied to us by them, and injected with 2 mg. of crystalline glutathione and 10 mg. of ascorbic acid for 5 generations.

We have observed that sexual maturation is retarded in rats maintained on the diet used in similar experiments by the Philadelphia Institute for Medical Research and that this deficiency may be corrected by supplementing the diet with yeast and wheat germ.

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A STRAIN DIFFERENCE IN RESPONSIVENESS OF CHICK THYROIDS TO THYROTROPIN AND A STEP-WISE INCREASE DURING THREE YEARS IN THYROID WEIGHTS OF CARNEAU PIGEONS

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WE HERE DESCRIBE A PROGRESSIVE step-wise change in the thyroid weight of young White Carneau pigeons studied during 5 years, and also a marked difference in the responsiveness of the thyroids of White Leghorn baby chicks which were obtained from two sources and which probably represent two hereditary strains. Since increase in the weight of the thyroid gland of various animals has become an accepted quantitative method for measurement of the thyrotropic content of anterior pituitary extracts the observations bear upon a common problem. These two types of thyroid variability indicate that quantitative assays of thyrotropin require the use of parallel tests with a standard preparation of thyrotropin. Collip has reviewed the earlier studies on thyrotropin (1). Smelser (2), Kabak and Liapin (3), Cope (4) and Bergman and Turner (5) have used both baby chicks and guinea pigs for thyrotropin assays.

Material used for study of a step-wise increase in the thyroid weight of Carneau pigeons. By means of selection Riddle (6) segregated and established races or strains of doves and of pigeons differing in thyroid weight. Our early work on thyrotropin was carried out on some of those races but only small numbers of young birds of such races were available for bioassay. In order to have larger numbers of birds of uniform age, and presumably of the same race, we began in 1936 the purchase of 6-week-old White Carneau pigeons[1] and since that time we have obtained a rather large and continuous supply of these birds. Within 10 days of their arrival the weights of the thyroids were obtained on all birds, including many which were used for the bioassay of prolactin. For the first 2 years, a satisfactorily uniform average thyroid weight of about 40 mg. was found and this encouraged us to make preliminary tests on their use for the quantitative assay of thyrotropin. Though the immediate results were satisfactory the introduction of the much less expensive baby chick as a test animal for this purpose by Smelser soon led us to discontinue the use of White Carneau for the assay of thyrotropin. In the autumn of 1938, however, we began a series of tests on the seasonal variation of the crop-sac response to prolactin (7) which was continued over a 2-year period; from these tests we obtained the weights of thyroids from nearly 300 additional young Carneau pigeons. Even at high dosage levels prolactin has no effect on thyroid weight in chick or in pigeons. We may therefore regard the weights obtained from pigeons injected with small doses of prolactin as control weights and place them alongside additional control weights of thyroids which were obtained from uninjected birds. Thyroids from 862 young Carneau pigeons are thus available for this part of the present study.

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[1] From the Palmetto Pigeon Plant, Sumter, S. C.

Results on a step-wise increase of thyroid weight in Carneau pigeons. The data are presented graphically in figure 1. The results are not treated statistically because the changes are great and are obviously significant; the coefficient of dispersion was slightly larger as the average weights increased. All points on the curve are averages of thyroid weights from groups of 10 pigeons unless an associated number indicates a larger group. When the data for a period of 5.5 years are viewed as a whole it is found that a marked and progressive increase in thyroid weight occurred during the last 3 years. From a fairly uniform average weight of 40 mg. in 1936–1937 an average of 179 mg. was attained in the winter of early 1941. An abrupt increase in the weight of the thyroids of quite young birds hatched during the autumn months of 3 successive years, successive increases which were not later wholly lost, is a remarkable result.

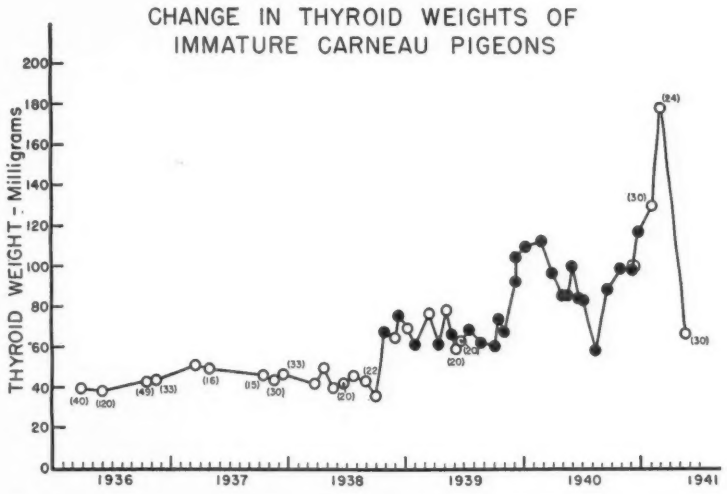

Fig. 1. Av. thyroid weight attained at 7 weeks after hatching, during a period of 5.5 years, in white carneau pigeons. Each point represents the value for 10 birds except as shown by a specified number. Solid points represent values from pigeons injected with low dosage (total of 2 mg.) of prolactin no. 657.

In mature doves and pigeons Riddle and Fisher (8) reported an annual cyclic change in thyroid weight with the maximum weight occurring in autumn and winter; for this period the thyroids of common pigeons averaged 48% heavier than in spring and summer. Those results were obtained on mature birds and relate to a succession of weight fluctuations annually. They are not directly comparable with the present data which relate to the weight which the thyroids of different groups of birds attained at 7 to 8 weeks after hatching—during the several seasons of 5.5 years. It is therefore of interest to note that during the last 3 years a seasonal difference in the attainment of thyroid weights occurred in these young birds which simulates, and is concurrent with, the changes in thyroid weights observed by others in parent birds.

Several laboratories in the Central and Eastern States now obtain their supply of pigeons for the assay of prolactin and certain other pituitary hormones from the same colony which supplied the birds described here. It is now obvious that White Carneau pigeons from this source and strain have been unsuitable for the bioassay of thyrotropin during the past 3 years. When control values in winter are double

those of summer it is not at all known whether a specified percentage increase of thyroid weight under dosage corresponds to equal or to quite unequal quantities of thyrotropin. In this material, during the past 3 years, only parallel tests with a standard preparation of thyrotropin would have provided a satisfactory quantitative assay of this hormone.

Strain difference in response to thyrotropin in chicks. Large racial or strain differences in sensitivity or response to anterior pituitary hormones have been described for 2 such hormones, namely for the bioassay of prolactin by crop-sac weight (9) and for the bioassay of the luteinizing hormone by weight of the seminal vesicles (10). We

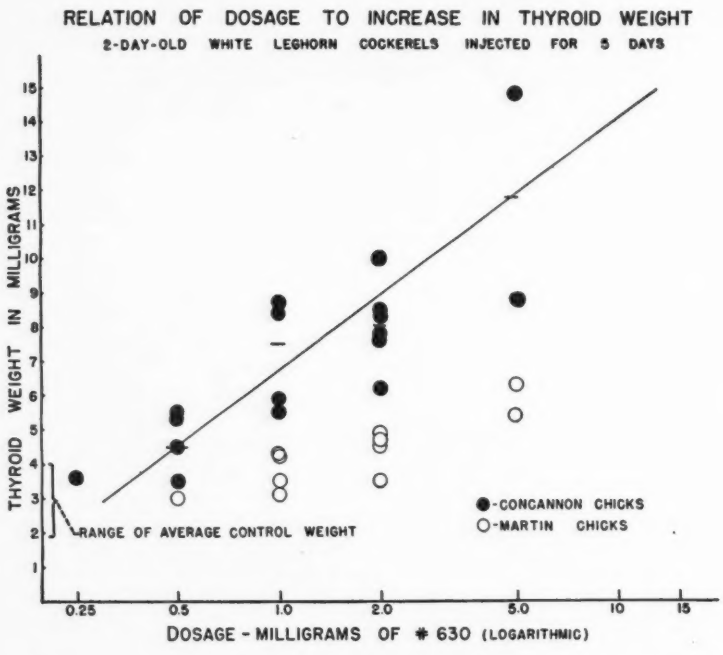

Fig. 2. Showing the marked difference in responsiveness of thyroids in 2 strains of chicks. Thyroid weights are plotted against logarithm of dosage and slope of line (see text) is for values thus plotted.

here report data which show that strain (or source) differences largely affect the bioassay of thyrotropin by thyroid weights of 2-day-old White Leghorn chicks. A local poultry dealer[2] provided us one supply of White Leghorn cockerels from a hatchery in Indiana. The other supply of White Leghorns we obtained directly from a different source in Indiana.[3] In all tests of these chicks for the response of their thyroids, as this is reported here, we used Smelser's method of thyrotropin assay which involves one subcutaneous injection daily for 5 days with autopsy on the sixth day.

In figure 2 the average weight of the thyroids of chicks from both strains are plotted against the total dosage of an isoelectrically soluble preparation (*no.* 630 = thyrotropic gonadotropic) which was made from cattle pituitaries. The average

[2] Concannon.
[3] Martin, of Ramsey, Indiana.

unstimulated thyroid weight varies from batch to batch of chicks but in this respect there was no appreciable difference between chicks from the 2 sources. The plotted data show that in order to produce an equal amount of stimulation the Martin chicks require four times as much thyrotropin as is required for the Concannon chicks. When chicks from these 2 sources were used for assay of adrenotropin, and when high dosage with fractions contaminated with thyrotropin was used, we further observed that the 2 types of chicks differ markedly in the maximum response which their thyroids may give during a 5-day period. The thyroids in Concannon chicks were found to increase in weight with increasing thyrotropin dosage until a

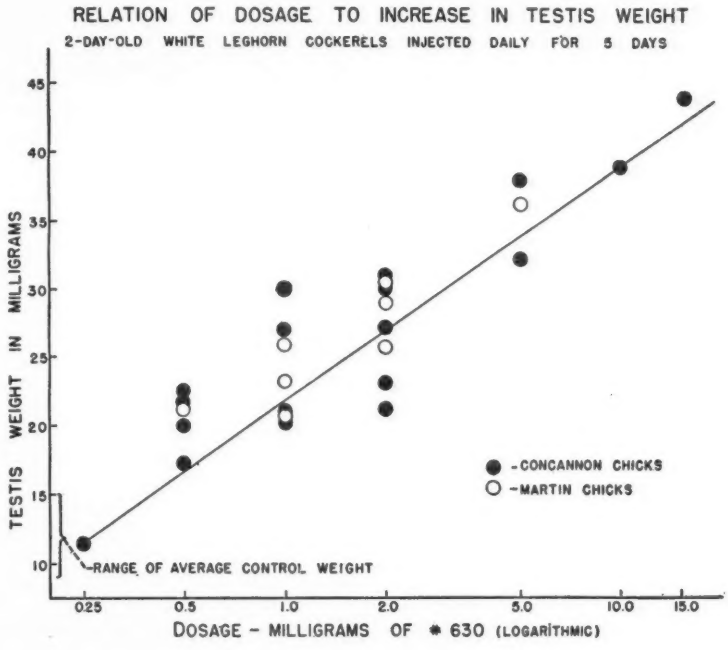

Fig. 3. SHOWING THAT THE TESTES OF THE 2 STRAINS OF CHICKS RESPOND EQUALLY TO PITUITARY GONADOTROPIN.

maximum weight of about 35 mg. is reached. In Martin chicks the maximum is only about 15 mg. (11). This difference in maximum organ weight attainable in a restricted period of days is similar to that found by us (9) for the crop-sac of different races of doves and pigeons treated with prolactin.

The slope of the straight line drawn through the data for Concannon chicks in figure 2 is 7.4; in other words, the thyroid weight increases by 7.4 mg. when the dosage is increased by a factor of 10. This relationship holds only when all the dosage is above the dosage for minimum stimulation and below the dosage which produces maximum stimulation. Using this same method Kabak and Liapin (3) obtained a slope of 6.3 which is probably not significantly different from our value of 7.4. We believe that this slope is a biological constant which will not vary with different strains of chicks when the same method and duration of treatment is used. Chicks

(such as those obtained from Martin) which increase the weight of their thyroids to only about 15 mg. while under a dosage that produces a maximum response give quantitative results only over a range of dosage extending from the threshold to 10 to 40 times above the threshold; but chicks like those obtained from Concannon should give quantitative results over a still greater range of dosage. It may be noted that either the type of chicks, or the method (4 daily injections), used by Bergman and Turner (4) was especially unfavorable in this respect, since a thyroid weight (7.3 mg.) in the region of maximum response for their chicks and conditions of injection was obtained with only 5 times the threshold stimulating dosage.

Weights of testes from the same chicks which were used for tests of thyrotropin (fig. 2) are shown in figure 3. The absence of a strain difference in responsiveness of the testes is apparent. It is further notable that chicks from these same two sources showed no difference in responsiveness of their adrenals to adrenotropic extracts (11). These facts seem to warrant the conclusion that the strain difference in thyroid responsiveness observed in our tests actually relates to a difference in their thyroids and not to a strain difference in the absorption, excretion or destruction of the injected extract.

DISCUSSION

Ratios for the relative sensitivity of the chick and guinea pig 'thyroid weight' methods were given by Smelser as 1 to 10, by Kabak and Liapin as 1 to 4, by Cope as 1 to 3, and by Bergman and Turner as 1 to 4, with the chick requiring least thyrotropin in all cases. Such ratios are of some practical value but their absolute magnitude is of no great significance when one realizes that 2 strains of chicks may differ in sensitivity by at least as much as a factor of 4 and that various strains of guinea pigs as well as other laboratory animals may be expected to show similar differences.

No adequate explanation has been obtained for the step-wise increase in thyroid size of young Carneau pigeons in the autumn of 1938, 1939 and 1940. Only slight changes[4] in the food-supply of the colony which supplied these young are said to have been made during the period covered by this study, and there has been no change in water supply since 1934. The 'grit' and salt mixture added to the food has not remained entirely constant[5] and perhaps this factor may be suspected. While in our hands these birds (since Jan. 1937) were fed the same grain and grit mixture which was currently used in the hatchery where they were reared. A genetic factor can not be excluded. The many birds which were actively serving as parents for the young received by us in 1936–1938 possibly included only a very few individuals with hereditarily large thyroids; larger numbers of such birds thereafter may unconsciously have been used for breeding purposes. The situation would require that such unconscious selection begin rather abruptly in the autumn of 1938 and be repeated in 1939 and 1940. It is not probable, however, that only genetic factors are involved.

Histological studies were made on very few of the thyroids from uninjected Carneau pigeons received from 1936 to 1938. In those few glands (weighing 30–60 mg. per pair) the epithelium is low and colloid is present in considerable amounts. In addition to their larger size the thyroids of uninjected control birds received in 1940 and 1941 have high or medium-high epithelium with occasional infolding; in the heaviest glands little or no colloid is present. Our histological studies thus indicate that the larger thyroids found in birds during the last 2 years were functionally more active than were the smaller thyroids of the birds received by us during 1936 to 1938. If this is true the basal metabolic rate of the birds received in more recent years should

[4] Kaffir corn from Kansas instead of Texas was used after early 1939.
[5] Less South Carolina sand during the last 2 years.

exceed that of the birds received earlier. Some data on this point are available. From 57 measurements made at 30° C. on uninjected birds in the earlier period and 34 similar measurements made in 1940 and 1941 it is found that the B.M.R. of the latter group averages 5.5% higher than that of the former group. This small difference, though probably significant and pointing in the direction expected, seems scarcely commensurate with either the difference in weight or with the apparent difference in histology of the 2 groups of thyroids.

SUMMARY

White Carneau pigeons, aged 6 weeks, were obtained from the same commercial hatchery over a period of 5.5 years and thyroid weights from 862 such birds were obtained 7 to 10 days later. During the first 2 years of observation the average weight of their thyroids was uniformly close to 40 mg. A step-wise increase in thyroid weight occurred in the autumn of 1938, 1939 and 1940 without complete return to previous summer levels and early in 1941 an average weight of 179 mg. was attained.

The source or cause of these changes is largely unknown. Incomplete histological data indicate that the later and larger thyroids were functionally more active. Ninety-one determinations of the B.M.R. of individuals of the 2 groups indicate an increase of only 5.5% in the group having larger thyroids.

During the last 3 years the thyroids of this widely used strain or type of pigeon have been unsuitable for the bioassay of thyrotropin though they earlier gave clear evidence of suitability.

White Leghorn 2-day-old chicks from 2 different sources in Indiana, and probably representing 2 hereditary strains, consistently gave quite dissimilar results when used according to the method of Smelser for the bioassay of thyrotropin.

Chicks from the one source require 4 times as much thyrotropin to produce an equal amount of stimulation, as this is measured by an increase of thyroid weight, as do chicks from the other source; and maximum stimulation during a 5-day test yields thyroids weighing only 15 mg. in the one type of chick and 35 mg. in the other more sensitive type.

Since chicks from these 2 sources showed no difference in their responsiveness to the gonadotropin and adrenotropin contained in various pituitary extracts it is highly probable that the observed strain or source difference in thyroid responsiveness relates to an actual difference in their thyroids and not to a strain difference in the rate of absorption, excretion or destruction of the injected extract.

Irrespective of the animal employed quantitative assays of thyrotropin require the use of parallel tests with a standard preparation of thyrotropin.

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THYROID-OVARIAN RELATIONS[1]

IV. EFFECT OF INTERMITTENT AND CONTINUOUS INJECTIONS OF ESTRONE ON HYPOPHYSEAL WEIGHT IN THYROHYPERPLASTIC ALBINO RATS

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IN A PRELIMINARY REPORT (1) dealing with the action of estrone on breast and genital tissues it was stated that large doses of estrone increased pituitary weight. This phenomenon, first described by Hohlweg (2) for normal rats, was subsequently confirmed by various investigators (3–7). Following publication of the report referred to it was discovered that certain dietary alterations had produced a thyroid hyperplasia in the animals used in this experiment. Further studies indicated that the presence of this condition had altered the response of the thyroid and ovaries to changes in the estrogen levels. It was demonstrated (8) that thyroid hyperplasia

TABLE 1. GROUPS AND TREATMENTS

Group	N	Treatment
Control	15	None
Intermittent administration	9	Received repeated (1–9) injections of 1.0 mg. estrone in oil every 10 days. Ten days after the last dose the animal was killed, and the pituitary weighed.
Continuous administration	14	Received repeated (12–109) injections of 0.1 mg. estrone in oil 6 times a week. The day following the last dose the animal was killed and the pituitary weighed.
Total	38	

was affected neither by castration alone nor by the administration of estrone[2] to castrates and non-castrates. However, castration with subsequent progesterone replacement resulted in a decrease of the hyperplastic state. This morphologic change was accompanied by a corresponding decrease in weight (9). It also was shown that 400-day-old sexually intact rats responded to moderate estrone treatment with ovarian changes indicative of pituitary failure (10).

In view of the above, the present study was made for the purpose of determining the effect of estrone on the pituitary weight of the thyrohyperplastic rat, as well as the relationship, if any, between the weight changes of this gland and the total dosage used, and the role played by the frequency of administration of estrogenic substance.

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[1] This research was supported in part by the Memorial Fund for Cancer Research and by the Rockefeller Fluid Research Fund of Stanford University.

[2] The estrone used in this study was supplied by Parke, Davis & Co., Detroit, Mich.

PROCEDURE

Thirty-eight mature female rats, all thyrohyperplastic, were divided into 3 groups and were treated as shown in table 1. The mean age of the animals was 418 days with a coefficient of variation of 14%. The variation was not considered serious and was not analyzed further, there being no noticeable evidence that age, within the general limits stated, affected the results.

The total dose of theelin received by each animal and the corresponding pituitary weights appear as a part of table 2.

TABLE 2. COMPARISON OF EFFECT OF INTERMITTENT AND CONTINUOUS DOSES OF ESTRONE ON PITUITARY WEIGHT

Intermittent					Continuous				
Unit dose = 1.0 mg. estrone every 10 days					Unit dose = 0.1 mg. estrone 6 times a week				
Total dose, (mg.)	Pituitary wt., (mg.)	Dev. from control mean[1] = x	Relative deviate = x/s[2]	P	Total dose, (mg.)	Pituitary wt., (mg.)	Dev. from control mean = x	Relative deviate = x/s	P
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)
1.0	12	−3.2	.69	.490					
					1.2	17	+ 1.8	.39	.697
					1.8	17	+ 1.8	.39	.697
2.0	15	−0.2	.04	.968					
					2.9	21	+ 5.8	1.25	.211
3.0	13	−2.2	.47	.638					
					3.5	28	+12.8	2.76	.006
4.0	14	−1.2	.26	.795					
					4.4	60	+44.8	9.64	.000
					4.5	32	+16.8	3.62	.000
5.0	14	−1.2	.26	.795					
					5.3	46	+30.8	6.63	.000
6.0	11	−4.2	.90	.368					
					6.6	24	+ 8.8	1.89	.059
7.0	17	+1.8	.39	.696					
					7.3	66	+50.8	10.94	.000
8.0	23	+7.8	1.68	.093					
					8.5	39	+23.8	5.12	.000
9.0	16	+0.8	.17	.865					
					9.1	48	+32.8	7.06	.000
					9.8	53	+37.8	8.14	.000
					10.4	35	+19.8	4.26	.000
					10.9	41	+25.8	5.55	.000

[1] Control mean = 15.20 mg., with a standard error of ±1.99 mg.
[2] s = Standard deviation of control distribution = ±4.64 mg.

ANALYSIS

Control pituitaries averaged 15.2 mg., with a standard deviation of ±4.6 and a standard error of the mean of ±1.2 mg. It is necessary to test the pituitary weights in each of the two treatments, after a variety of total dosages against the mean of the control group. The conventional method of comparison was followed. For each total dosage for each rat the deviation from the control mean was taken, and converted from mg.-units into sigma-units, that is, into relative deviates. From these, the probability was calculated that a difference as large or larger than that found in either direction, could have occurred by chance alone. As a measure of significance, the usual criterion of $P = .05$ or less was set up. The statistics appear in table 2.

None of the weights in the intermittent group differed significantly from the control mean, for all deviations noted could have been the result of chance. Therefore, it may be concluded that estrogen in large interrupted doses did not affect the weights of the rat pituitaries to any marked degree. On the other hand, with one exception, the continuous administration of estrone resulted in highly significant deviations throughout the entire group after the 3 mg. cumulative dose level had been reached. The one exception noted was that animal which failed to show a pituitary weight increase after the injection of 6.6 mg. The probability corresponding to this latter value is .059, hence not significant by the criterion established. However, as it may be assumed from the figures that the administration of estrone in con-

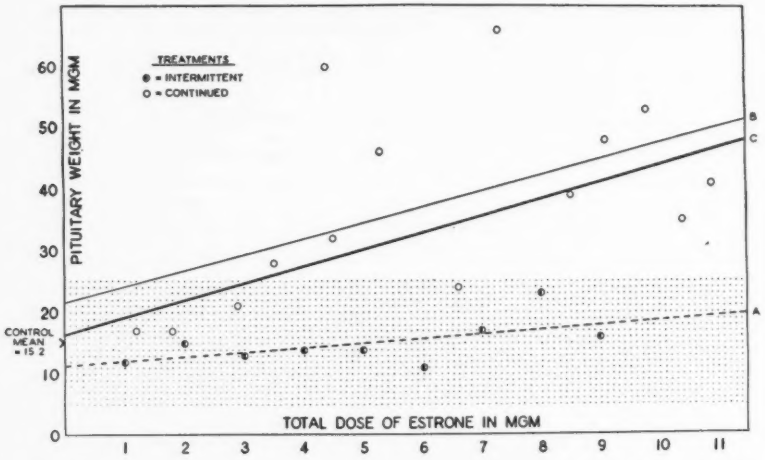

Fig. 1. PITUITARY WEIGHTS IN RATS FOLLOWING ESTRONE ADMINISTRATION. A, Intermittent treatment. B, Combined treatment (original data). C, Combined treatment (adjusted data). The stippled area represents the fiducial limits of the control mean at the 5% level.

tinuous doses does increase pituitary weight, all of the probabilities may be halved, as only one tail of the normal frequency curve comes into consideration. Thus the deviations in animals having received a total dose of 3.0 mg. or more are all highly significant, and it may be stated that the increase in pituitary weight following the administration of estrone in the doses used is a real and definite one.

The foregoing, however, gives no information as to any possible correlation between the pituitary weight and the total dose of estrone administered. Therefore, the individual observations have been plotted in figure 1, and the regression lines added. It will appear below why two such lines have been calculated for the continuously treated group.

The constants are given in table 3. The calculations were made to more places than appear in the table.

The symbols in table 3 are self-explanatory, but it may be emphasized that the standard error of estimate (s_e) is that part of the total variation (sY) remaining, after the portion due to the regression itself has been removed. In other words, it is a more accurate estimate of the experimental error.

Intermittent treatment. As was expected, the regression of pituitary weights on the total dose administered was very small ($b = .75$), all of the points falling within

the fiducial limits at the 5% point of the control mean (i.e., 15.2 ± 9.1 mg.). The correlation coefficient, r, is +.58, which, while not statistically significant ($P = .101$), does indicate a certain degree of association.

The mean pituitary weight of the group was 15.0 mg. with a standard deviation (sY) of ±3.5 mg., which latter is reduced by 13%, giving a standard error of estimate of ±3.1.

Continued treatment. The regression coefficient is +2.6 with a standard error of ±1.2. The coefficient of correlation is +.54, which is barely significant, the probability being .048. The mean weight of the group was 37.6 mg. with a standard deviation of ±15.6 mg. which is reduced by 12.3% through linear regression, the standard error of estimate being ±13.7.

The two outlying values at $X = 4.4$ and 7.3 respectively, are unexplained. The

TABLE 3. REGRESSION AND CORRELATION STATISTICS

Statistics	Symbol	Control	Treatment		
			Intermittent	Continuous	
				Original	Adjusted
(1)	(2)	(3)	(4)	(5)	(6)
Number	N	15	9	14	12
Mean total dose, mg.	$\bar{X}$		5.00	6.16	6.21
Mean pituitary weight, mg.	$\bar{Y}$	15.20	15.00	37.64	33.42
Standard deviation	s_Y	±4.64	±3.54	±15.63	±12.28
Standard error	$s_{\bar{Y}}$	±1.20	±1.18	± 4.18	± 3.54
Regression coefficient	b		+ .75	+ 2.59	+ 2.75
Standard error	s_b		± .40	± 1.17	± .70
St. error of estimate	s_e		±3.08	±13.71	± 8.09
% Reduction of s_Y	$s_Y - s_e / s_Y$		13.01	12.31	34.11
Correlation coefficient	r		+ .581	+ .539	+ .778
Significance of r	P		.101	.048	.005

former lies outside the 5% fiducial limits (+26.9 mg.) of the regression line, and the latter just within. On the supposition that the large deviations of these two pituitary weights from the regression line were caused by some individual and unknown factor inherent in the animal itself, and were thus aberrant, they were omitted, and a new regression line calculated.

The mean weight now becomes 33.4 mg. with a standard deviation of ±12.3. It will be noted that the new regression coefficient, 2.7 is practically the same as the old one, but a lower level. Thus the omission of the two questionable values appears justified in that a more accurate estimate of the true relationship existing between the two variables may be made.

With this adjustment, the correlation coefficient rises from +.54 to +.78, with a corresponding decrease in the probability of occurrence by chance from .048 (barely significant) to .005 (highly significant). The standard error of estimate is also reduced from ±13.7 to ±8.1, the latter showing a 34.1% decrease when compared to the corresponding standard deviation.

Using the adjusted regression coefficient as a basis for prediction, it may be stated that, on the average, for each milligram increase in total estrogen dosage there is an increase in the weight of the pituitary gland of 2.75 mg.

Our results indicate that continued estrogenic treatment of thyrohyperplastic rats leads to an increase in weight of the pituitary gland. We estimated the average

increase to be 2.75 mg. for each added milligram of estrone. Wolfe and Wright (11), using normal and castrated females, and Weil and Zondek (12), using normal males and females, have reported similar findings.

Intermittent administration of comparative amounts of estrone failed to affect pituitary weight to a significant degree. We believe this to be due to the absence of a cumulative effect (fig. 1).

It is therefore of importance to note that equal total doses of estrone given over equal periods of time produced different weight responses in the pituitary gland, the difference being a function of the frequency of administration.

SUMMARY

Intermittent doses of estrogen, administered at 10-day intervals, to thyrohyperplastic rats did not affect pituitary weight to a degree which is statistically significant by the criterion set up and by the limits of the experiment. It is indicated, however, that there was some slight increase in pituitary weight with increasing total doses.

Continuous doses of one-tenth the size, administered 6 times a week, to thyrohyperplastic rats resulted in an increase in pituitary weight of a highly significant order, and furthermore, within the limits of the experiment, the increase was proportional to the total dose.

The quantitative action of an estrone (theelin) on the pituitary weight of thyrohyperplastic female rats depended primarily on the frequency of administration.

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HORMONAL ACTION OF THE TOAD HYPOPHYSIS ON MAMMALS

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THE ACTION OF THE HORMONES of the hypophysis of the toad *Bufo arenarum* Hensel upon members of the same species has been well studied (1–3). The action of the various hormones in mammals has been studied also (4–8). These have included the diabetogenic, growth, thyrotropic, adrenotropic gonadotropic and lactogenic hormones, and in addition, factors affecting the weight of the thymus and spleen. The technical details and a more complete bibliography may be found in the references referred to (4–8).

General Technic. A total of some 2,000 hypophyses of the toad *Bufo arenarum* Hensel which were recently captured were used. The hypophyses were removed by the buccal route. The two lobes[1] were allowed to dry on separate watch glasses until the time of injection when they were suspended in a 0.8% solution of saline.

The following animals were used as receptors: hypophysectomized dogs in which only 4 gm. of pancreas remained post-operatively, guinea pigs, chickens, pigeons, and normal and hypophysectomized rats. The rats weighed approximately 100 gm. and were operated upon according to the original technic of Smith (9–12) as modified by Selye. Injections twice daily were begun on the 15th post-operative day and were continued for 14 days at which time the animals were killed. Autopsy was performed to determine if hypophysectomy had been complete. If incomplete, the data were discarded.

The experiments were performed between August 1 and October 30, that is to say between the end of winter and the beginning of summer.

Diabetogenic action of toad hypophyses

The principal lobe (pars distalis) of the pituitary of the toad has a diabetogenic action on the toad (13). Similarly pituitaries of fish, batrachians, birds, and mammals (among them man) are diabetogenic in the toad (14). However, the diabetogenic action of the pituitary of the toad on mammals has not been demonstrated previous to the present work.

Methods. Hypophysectomized dogs in which the pancreas had been surgically reduced to 4 gm. in the vicinity of the main duct, were injected intraperitoneally for 4 days with a suspension of the anterior hypophysis (pars distalis) of the toad *Bufo arenarum* Hensel. The untreated dogs appear normal and have a normal blood sugar yet they are extremely sensitive as test animals for revealing the diabetogenic factor (15). Before and during the period of injection the fasting blood sugar in the capillary

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[1] The principal lobe of the toad hypophysis (pars distalis or pars chromophila) corresponds physiologically to the anterior lobe of mammals but in the toad as in the other anurans, it is situated behind the neuro-intermediary lobe (pars intermedia plus pars neuralis) which corresponds functionally to the posterior lobe of mammals.

blood of the ear was determined as an index of the activity of the suspensions employed. Blood sugars were determined by the method of Hagedorn and Jensen with previous precipitation by Somogyi's method. As controls, suspensions of fresh liver and kidney from toads were injected.

Results. *Dog 610* was sensitive to the daily injection of 20 mg. of fresh bovine anterior pituitary suspension per kg. of body weight and showed frank hyperglycemia following the daily injection of 9.6 and of 19.2 mg. of the anterior pituitary of the toad, but no response to the injection of 4.8 mg. of the suspension. *Dog 607*, recently sensitive to the daily injection of 40 mg. of a suspension of bovine anterior

TABLE 1. BLOOD SUGAR IN HYPOPHYSECTOMIZED DOGS WITH ONLY 4 GM. OF PANCREAS. Injected intraperitoneally for 4 days with suspensions of toad pituitary tissue. Fasting capillary blood sugar from the ear. Deproteinization by the method of Somogyi and determination by the method of Hagedorn and Jensen.

Dog 610—8.6 kg.								*Dog 607*—10.1 kg.							
Toad ant. pit.			Blood sugar in mg. % after days					Toad ant. pit.			Blood sugar in mg. % after days				
No. of lobes	Mg. per kg. per day							No. of lobes	Mg. per kg. per day						
	Fresh	Dry	0	1	2	3	4		Fresh	Dry	0	1	2	3	4
50	4.8	0.8	71	102	106	105	115								
100	9.6	1.6	112	153	122	146	283	100	8.2	1.4	96	107	96	129	131
200	19.2	3.2	70	103	182	328	297								
Pituitary lobe (pars distalis) of bovine hypophysis															
	20	4	76	172	259	211	247		20	4	103	101	95	82	119
	40	4	104	238	221	236			40	8	111	138	169	240	
Toad liver *Dog 627*, 8 kg.								Toad kidney *Dog 626*, 11 kg.							
	21		78	79	80	86	87		14		78	79	85	73	89

pituitary, showed slight hyperglycemia following the injection of 8.2 mg. of the anterior hypophysis of the toad. In *dog 610*, therefore, the effective dosage of toad pituitary was found to be between 4.8 and 9.6 mg. per kilo per day and in *dog 607* slightly more than 8.2 mg. A comparison of the activity of toad and bovine pituitaries in terms of weight of the fresh gland, shows that the toad pituitary is 2 to 4 times more active in the wet state, while in the dry state it is 5 times greater than that of the bovine pituitary.

The injection of suspensions of toad liver and kidney in the same dosage did not change the blood sugar (table 1).

Conclusions. The principal lobe (pars distalis) of the hypophysis of the toad has a specific diabetogenic action[2] on the hypophysectomized dog with only 4 gm. of intact pancreas. This action is 2 to 4 times greater than that produced by an equal weight of the anterior pituitary of cattle.

Growth action of toad hypophysis

The anterior pituitary (pars distalis) of anuran batrachians accelerates growth and causes the metamorphosis of the larvae of the same animals (9, 16, 17). Similarly, ex-

[2] In referring in this paper to specific actions of the pituitary we mean that these actions are not obtained with extracts of other organs of the same animal used under similar conditions.

tracts of the anterior lobe of cattle increase the growth of hypophysectomized tadpoles (12). The action of the hypophysis of batrachians on mammalian growth has, however, not been studied.

Methods. Hypophysectomized rats weighing approximately 100 gm. were injected with suspensions of toad pituitaries. Two weeks after hypophysectomy, the rats were divided into 4 groups. *Group 1* was injected with the anterior pituitary (pars distalis) of the toad, *group 2* with the posterior pituitary (neuro-intermediate lobe), *group 3* with suspensions of various tissues of the toad, and *group 4* with Ringer's solution. A fifth group consisted of normal untreated rats of similar weight.

TABLE 2. BODY WEIGHT AND LENGTH OF FEMALE HYPOPHYSECTOMIZED RATS AFTER 14 DAYS' TREATMENT WITH PREPARATIONS OF TOAD PITUITARY EXTRACTS. The figures represent the average for the animals in each group. Autopsy on 15th day of injection.

Group	No. of rats	Lobes injected			Weight of animals			Length	
		No.	Fresh	Dry	Before inject.	After inject.	Total wt. change	Body	Tail
					gm.	gm.	gm.	cm.	cm.
Normal rats	4	—	—	—	—	94	—	27.2	12.4
	4	—	—	—	99	126	+27	29.2	12.7
Hypophysectomized rats									
(a) control	9	—	—	—	96	90	− 6	27.3	11.8
			mg.	mg.					
(b) pars distalis	3	1	3.3	0.55	81	91	+10	26.5	12.0
	3	3	9.9	1.65	88	106	+18	27.5	12.0
	3	5	16.5	2.75	100	130	+30	29.3	12.5
	3	10	33.0	5.50	109	129	+20	30.5	13.8
(c) neurointermediary	3	5	10.0	1.85	79	84	+ 5	25.5	11.1
	2	10	21.0	3.70	100	100	± 0	27.7	13.1
			mg. tissue						
(d) muscle	3		27		93	93	0	27.3	12.0
(e) kidney	3		26		100	97	− 3	28.1	12.0
(f) liver	3		24		96	92	− 4	27.8	12.2

The rats were weighed every other day. The animals were killed on the 15th day of the injection and the total length and that of their tails were measured. These experiments, carried out on 43 females, were repeated and confirmed with 13 males.

Results. Each group consisted of at least 3 rats and the results are expressed as the average for each group.

Rats of *group 1* received daily injections of 3.3, 9.9, 16.5, and 33 mg. of the anterior lobe (pars distalis) of toad hypophyses. The body weight began to increase at once, after treatment was begun and continued progressively to the end. This increase depended on the dosage and reached its maximum with 16.5 mg. daily, beyond which it declined perhaps because of the toxic effect of the injected proteins. The increase in weight caused by the injection of 16.5 mg. per day was equivalent to the spontaneous growth of normal rats of equal initial weight. The growth unit fixed by Collip[3] was found to lie between 3.3 and 9.9 mg. (which would be equivalent to 1 to 3 lobes), these amounts causing an increase of 10 and 18 gm. respectively. Two lobes would therefore contain 1 Collip growth unit and 150 units would be contained in 1 gm. of

[3] Daily dose of anterior lobe preparation which causes an increase of 14 gm. of body weight in 14 days in these rats.

fresh toad anterior pituitary gland. The increase of weight noted corresponds to actual growth since there was an increase in the total length of the skeleton, of the neck, and also of the teeth (table 2, fig. 1).

The hypophysectomized rats of *group 2* were divided into 2 subgroups receiving 10 and 21 mg. daily of a suspension of the posterior lobe of the pituitary (neuro-intermediary lobe). The first subgroup showed a slight increase of 5 gm. in weight which was difficult to interpret because it was not accompanied by a corresponding increase in the length of the bones. The second subgroup maintained its weight with slight fluctuations. The external appearance of these animals in their lack of activity, their hypotonicity and their lustreless skin resembled that of the hypophysectomized controls of the third group, and differed strikingly from that of the group injected with anterior lobe extract, for these animals appeared normal.

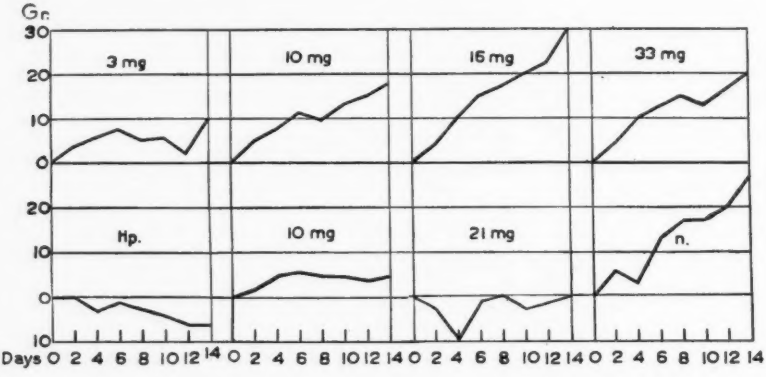

Fig. 1. Change in body weight of 100 gm. female rats. Each curve represents the average for 3 or more animals. Injections were made for 14 days. Numbers represent mg. of fresh gland injected per day. *Upper curves*, Hypophysectomized rats injected with suspension of pituitary lobe of the toad (pars distalis). *Lower curves*, Hp = Hypophysectomized rats not injected. Ten mg. and 21 mg. = hypophysectomized rats injected with suspension of the neuro-intermediary pituitary lobe of toads. *n* = normal untreated controls.

In all of the rats of *group 3*, injected with Ringer's solution (the vehicle of all the solutions employed) and of *group 4* which received an amount of liver, kidney, and muscle suspension of the toad equivalent to that of the hypophysis, the body weight diminished.

Conclusions. The anterior lobe of the hypophysis (pars distalis) of the toad has an intense and specific action on the growth of hypophysectomized rats. The maximum effect has been obtained with a dose of 16.5 mg. of fresh gland daily. Each anterior lobe contains 0.5 Collip growth units and 1 gm. of fresh tissue contains 150 units. The posterior lobe (neuro-intermediary lobe) has but slight growth action on the hypophysectomized rat in the amount of 10 mg. per day.

Thyrotropic action

In the toad *Bufo arenarum* Hensel the hypophysis develops and maintains the anatomical and functional state of the thyroid (18). The action of the anterior pituitary of the toad on the thyroid of the guinea pig has been studied by Houssay, Novelli, and Sammartino (19) who demonstrated a positive but weak action. Stimulation of the thyroid in mice and guinea pigs by the pituitary of *Rana pipiens* has been demonstrated (1, 3).

Methods. Various doses of suspensions of the anterior pituitary (pars distalis) of the toad were injected into normal chickens, one day old, normal guinea pigs weighing 150 gm., immature rats 21 days old and hypophysectomized female and male rats weighing 100 gm. The weight of the thyroid gland was determined after one or more days of treatment.

In some rats of *groups* 3 and 4 and in the guinea pigs, the effect of the injected posterior lobe (neuro-intermediary lobe) of the pituitary of toads on the thyroid was studied. Controls were used in all of the experiments.

Results. In the 4 groups of animals injected with anterior lobe (pars distalis) suspensions, the weight of the thyroid was greater than in the controls (table 3, fig. 2). In the chickens, immature rats, and especially the hypophysectomized rats, it was observed that a larger dose of anterior lobe extract produced a greater weight of the

TABLE 3. AVERAGE OF GLAND WEIGHTS AND % WEIGHT CHANGES IN FEMALE HYPOPHYSECTOMIZED RATS TREATED FOR 14 DAYS WITH TOAD PITUITARY EXTRACTS

Group	Absolute weight in mg.					Weight per 100 gm. of rat					% wt. increase over hypophysectomized controls					
	thyr.	adrn.	thym.	spln.	ovary	thyr.	adrn.	thym.	spln.	ovary	body wt.	thyr.	adrn.	thym.	spln.	ovary
						mg.	mg.	mg.	mg.	mg.						
Normals untreated	13	23	269	631	30	14	24	286	671	32						
	17	33	350	808	42	14	26	280	646	33						
Hypophysectomized (a) controls	5	9	174	404	11	6	10	193	449	13						
(b) pars distalis inj.	10	12	179	427	8	11	13	196	469	9	1	82	28	3	5	−32
	11	14	236	514	17	10	13	222	485	16	18	100	49	35	27	44
	16	16	—	—	14	12	12	—	—	11	44	191	70	—	—	19
	12	15	327	805	16	9	12	253	624	12	43	118	60	88	99	36
(c) neurointermediary lobe injected	6	11	82	312	8	7	13	98	371	10	0	9	17	−52	−22	−32
	9	13	145	629	16	9	13	145	629	10	10	73	38	−17	56	36
(d) muscle inj.	5	9	147	389	9	5	9	158	418	10	3	−10	− 4	−16	− 3	−24
(e) kidney inj.	10	10	170	352	17	10	10	175	363	17	7	82	4	− 2	−12	44
(f) liver inj.	6	7	130	330	17	7	8	141	359	18	2	10	−25	−25	−18	44

thyroid. This effect was specific since it was not produced with extracts of other organs of the toad. In the treated hypophysectomized rats, the weight of the thyroid reached that of normal rats of the same body weight. The maximum weight was obtained with a daily dose of 16.5 mg. of the anterior lobe of the pituitary; beyond this dosage level it decreased. Similar changes were observed in the body weight which increased progressively with the amount of anterior lobe extract injected, to a maximum of 16.5 mg. daily, and then diminished. It might be thought that the variation of the weight of the thyroid was non-specific and followed changes in the body weight of the animal. This is not the case, however, because in the hypophysectomized animals that received different doses of suspensions of the anterior lobe of toads the curve of the percentage increase of the weight of the thyroids in relation to the weight of the thyroids of the untreated controls is much greater than that of similar values obtained for the adrenal and body weight (tables 2 and 3, fig. 3). In addition, the weight of the thyroid calculated per 100 gm. of rat was greater than that of the thyroid in the hypophysectomized untreated controls, and also that of animals which received extracts of other tissues of the same animal (muscle, liver, kidney) (table 3, fig. 2).

The injection of a suspension of the posterior lobe (neuro-intermediary lobe) of the pituitary of the toad did not modify the weight of the thyroid in the guinea pigs, but caused a slight increase in the weight of the thyroids of immature mice. In the hypophysectomized rats, the weight of the thyroids was increased although not to the

same extent as in rats receiving anterior lobe injections. This action appears to be specific because the % increase in weight was greater than in the untreated controls, and a weight increase was not observed with suspensions of other organs of the same animal (muscle, liver); one exception was that observed with suspensions of kidney, which could be assigned to contamination with the adrenal (table 3, fig. 2). Every

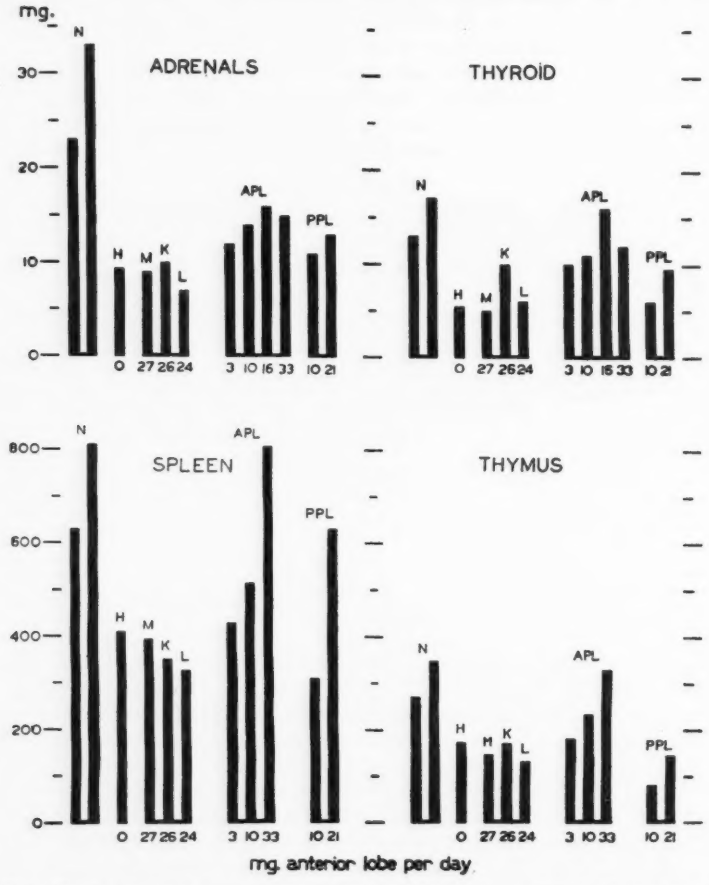

Fig. 2. GLAND WEIGHTS IN EXPERIMENTAL ANIMALS. Each black column represents the average weight of the adrenals, thyroid, spleen and thymus of 3 or more 100 gm. female rats. N = normal controls weighing between 94 and 125 gm. which are the extreme limits of weight of the rats of the other groups. Hypophysectomized rats injected with H: Ringer's solution; APL: suspension of principal lobe (pars distalis) of hypophysis of toad; PPL: suspension of neuro-intermediary lobe of hypophysis of toad; M: muscle suspension; K: kidney suspension; L: liver suspension of toad.

possible care was used in separating the posterior lobe to avoid contamination with anterior lobe tissue.

Conclusions. The anterior lobe (pars distalis) of the hypophysis of the toad has an intense thyrotropic action on chickens, guinea pigs, immature rats and hypophysectomized rats. This action is specific, varies with the dose employed, and exceeds the adrenotropic action.

The posterior lobe (neuro-intermediary lobe) of the hypophysis of the toad does not have a thyrotropic action in the guinea pig, has a slight action in the immature rat, and a pronounced action on the hypophysectomized rat. Regarding the latter, the action is specific but much less than that of the anterior lobe of the pituitary.

Adrenotropic action

The anterior pituitary (pars distalis) governs the development and maintenance of the adrenal cortex in batrachians (10, 12). Adrenotropic extract of mammalian pituitaries stimulates the adrenal cortex of batrachians (20–23). This is the first study of the action of the anterior pituitary gland of batrachians on mammalian adrenals.

Methods. The adrenotropic action of the anterior (pars distalis) and posterior (neuro-intermediary lobe) pituitary of the toad *Bufo arenarum* Hensel has been tested on normal 150 gm. guinea pigs, 21-day-old female rats and 100 gm. hypophysectomized female rats. These animals, divided into groups, received varying doses of anterior and posterior lobe suspensions, suspensions of other organs of the toad, and Ringer's solution. After several days of treatment the animals were killed in order to extract and weigh the adrenals.

Results. The injection of anterior pituitary extract of the toad caused a definite increase in the weight of the adrenals of all the animals studied—guinea pigs, 21-day-old rats, and hypophysectomized rats. In 21-day-old rats, this effect was augmented by adding pregnancy urine. There was a relationship between the amount of anterior lobe extract injected and the observed increase in the weight of the adrenals. This was also noted in the immature and hypophysectomized rats. In the latter group the maximum response was reached with a dose of 16.5 mg. daily beyond which a diminution was observed (tables 2 and 3, fig. 2).

This adrenotropic action of the anterior lobe of the pituitary of the toad is specific. It is independent of the action that the same extract exerts on the growth of the hypophysectomized rat because it is not produced with extracts of other organs (kidney, liver, muscle), it occurs with doses smaller than is required to promote growth and in all dosage levels of anterior pituitary extract, the % increase in the weight of the adrenals was greater than that of the body, although it was less than that of the thyroids (fig. 3). Furthermore, if the weight of the adrenals is calculated per 100 gm. of rat, the weight is greater in the treated than in the control animals.

The posterior pituitary lobe extract of the toad had no adrenotropic action in guinea pigs, but it had an action in 2 of the 3 immature rats studied. There was a definite action in hypophysectomized rats although to a less degree than that of the anterior lobe. For the same reasons as for the anterior lobe extract, this action must be considered specific because the two lobes were separated with all possible precautions to avoid accidental contamination.

Conclusions. The anterior lobe (pars distalis) of the hypophysis of the toad *Bufo arenarum* Hensel has a specific adrenotropic action on the normal guinea pig, the immature rat, and the hypophysectomized rat. This action increases with the dosage, is less strong than the thyrotropic and is stronger than the growth action.

The posterior lobe (neuro-intermediary lobe) of the hypophysis of the toad has a specific adrenotropic action on the hypophysectomized rat although its action is less intense than that of the anterior lobe. This action increases with an increase of the dosage.

Action on thymus and spleen

The action of the anterior lobe of the hypophysis (pars distalis) of batrachians on the thymus and spleen of mammals has not been studied heretofore.

Methods. Male and female hypophysectomized rats were injected for 2 weeks with various doses of suspensions of the anterior and posterior lobes of the hypophysis of the toad *Bufo arenarum* Hensel. At the end of this period they were killed and the thymus and spleen were weighed. As controls, untreated hypophysectomized rats, and normal and hypophysectomized rats were injected with extracts of muscle, kidney, and liver of toads.

Results. The thymus of hypophysectomized rats injected with anterior lobe suspension increased in weight until it reached that of the normal controls with a dose of 33 mg. daily. This increase varied directly with the amount injected and was specific and independent of the growth action for the same reasons as those given for the adrenotropic action (tables 2 and 3, fig. 2).

The thymus of hypophysectomized rats injected with posterior lobe extract presented no significant variations in weight although in the females it was slightly less and in the males slightly more than in the controls.

Changes in the weight of the spleen of hypophysectomized rats injected with anterior and posterior lobe suspension are analogous to those in the thymus, the only difference being that the weight of the spleen increased in those animals receiving the maximum dose of posterior lobe suspension (tables 2 and 3, fig. 2).

Conclusions. The anterior lobe (pars distalis) of the hypophysis of the toad *Bufo arenarum* Hensel has a specific and intense action on the weight of the thymus and spleen of hypophysectomized rats. The degree of this action varies with the dose administered.

The posterior lobe (neuro-intermediary lobe) has no action on the weight of the thymus of the hypophysectomized rat, but in large doses the weight of the spleen increases.

Gonadotropic action

The anterior lobe (pars distalis), of the pituitary governs the development and maintenance of the sex glands of batrachians as well as of vertebrates. In the toad *Bufo arenarum* Hensel it has been observed that hypophysectomy in the male causes testicular atrophy (24, 25) and in the female involution of the oviduct (26). Furthermore, implantation of the anterior lobe of the same species leads in the male to testicular stimulation and to the appearance of the clasping reflex and in the female to ovulation and to the expulsion of ova (27–29).

Ovulation in female batrachians does not occur or is difficult to obtain by the administration of pituitary extracts of other species such as fish, reptiles, birds, and mammals (26, 28, 30, 31). There is a zoological specificity, relative or quantitative, in the gonadotropic action of the hypophysis.

The gonadotropic action of the anterior pituitary of batrachians on mammals has been studied particularly in the mouse. Negative results have been observed by Lipschütz and Paez (32), Martins (33), Magistris (34), Zondek (35), Adams and Tuckey (3) Benazzi (30, 36) and positive results by Zawadovsky et al. (37), Zwarenstein (38), Adams and Granger (1, 2).

Methods. The gonadotropic action of the anterior and posterior lobe of the hypophysis of the toad was tested out on mammals by injecting extracts in increasing doses to rats, then killing them and weighing the sex glands (ovaries, uterus, testicles, prostates). Controls consisted of untreated rats and rats injected with human pregnancy urine or with suspensions of various organs of the toad (muscle, kidney, liver).

The rats were divided into 4 groups as follows *group 1*, day-old females; *group 2*, 21-day-old females injected with human pregnancy urine; *group 3*, hypophysectomized females; and *group 4*, hypophysectomized males.

Results. Suspensions of the anterior lobe of the hypophysis of the toad were injected into animals in each of the 4 groups, in amounts varying between 3.3 and 220 mg. per rat per day. The only significant variation noted was the increase in the weight of the uterus of the rats of *group 2* which received pregnancy urine together with the anterior lobe suspension. In the hypophysectomized females, the increase in weight of the ovaries of those injected with anterior lobe extract was analogous to

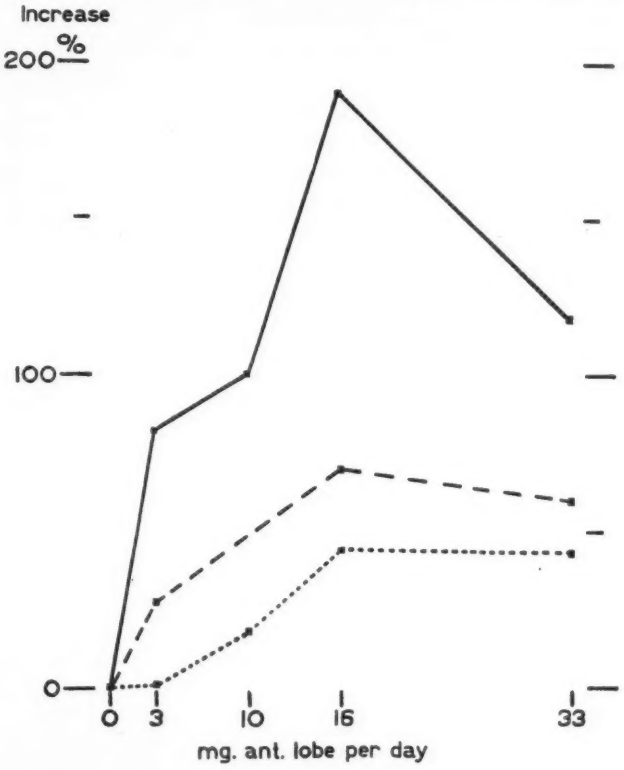

Fig. 3. FEMALE HYPOPHYSECTOMIZED RATS INJECTED WITH SUSPENSION OF PRINCIPAL LOBE (PARS DISTALIS) OF TOADS FOR 14 DAYS. Per cent increase in relation to those injected with Ringer's solution in the weight of the thyroid (————), adrenals (- - - - - -), and body (· · · · · ·). The average of a group of 3 or more rats has been used for each determination.

that of those injected with extracts of other organs of the toad (muscle, kidney, and liver) which was an absolute, as well as a relative increase (tables 2 and 3). In the hypophysectomized male, the slightly greater weight of the testicles, the coagulating glands, and the prostate and seminal vesicles was due to the growth action of the extracts because the weight of these organs per 100 gm. of rat was the same in those which received anterior lobe extract as it was in the untreated controls and in the controls which received extracts of other organs. In addition, the % increase in weight of the same organs in rats injected with anterior pituitary lobe extract (over and above the weight of the untreated hypophysectomized rats) was no greater than the increase of the body weight of the animals calculated in the same manner (tables 2 and 3).

Posterior lobe extract was injected into rats of *groups* 1, 3, and 4 in doses which varied between 10 and 88.2 mg. of the fresh gland per day. In none of the rats did the weight of the ovary or uterus change significantly.

Conclusions. The anterior pituitary lobe (pars distalis) of the toad *Bufo arenarum* Hensel has no apparent gonadotropic action on immature female rats or on hypophysectomized adult male and female rats. In three immature rats the injection of anterior lobe extract of the toad augmented the increase of weight of the uterus produced by the injection of human pregnancy urine.

The posterior pituitary lobe (neuro-intermediary lobe) does not have a gonadotropic action on immature female rats or on male and female hypophysectomized rats.

Prolactin action

Leblond and Noble (39) implanted the hypophysis of mammals, birds, reptiles, amphibians, and fishes in the dermis of the skin which covers the crop of pigeons and obtained thickening of the mucosa of the crop. They do not believe this action to be specific for the 3 latter species since they obtained similar thickening with extracts of liver of pigeons, turtles, and fish.

Methods. The presence of prolactin in the anterior lobe (pars distalis) of the hypophysis of the toad *Bufo arenarum* Hensel was sought by injecting extracts of this lobe into young pigeons and studying the changes produced in the crop gland. In one group of animals, subcutaneous injections were made at a distance from the crop. The increase in the weight was taken as an index of activity. In another group, the injections were made locally into the dermis of the skin which covers it, and subsequently noting any thickening produced in its wall (method of Reece and Turner (40)). Controls consisted of young pigeons injected with Ringer's solution or with extracts of liver, kidney, or muscle of the toad.

Results. In the young pigeons of *group* 1 injected subcutaneously with 33 or 76.5 mg. of anterior lobe suspension daily, no significant changes in the weight or histology of the crop could be noted.

In the young pigeons injected intradermally with 33 mg. or more of anterior lobe extract daily a definite localized thickening of the mucosa of the crop occurred which was confirmed by histological examination. This thickening was not produced by the daily injection of 100 mg. of suspensions of other organs of the toad (liver, kidney, and muscle). Nevertheless, this thickening was not of the magnitude produced by 1 unit of prolactin by Reece and Turner (40).

Conclusions. The anterior lobe (pars distalis) of the hypophysis of the toad *Bufo arenarum* Hensel has a slight prolactin-like action on the crop of the pigeon.

Conclusions

The principal lobe (pars distalis) of the hypophysis of the toad *Bufo arenarum* Hensel has definite hormonal actions when injected into mammals. In the order of decreasing intensity these are diabetogenic, growth, thyrotropic, adrenotropic, increase in weight of thymus and spleen, and prolactin. A gonadotropic action in mammals was not demonstrated for the toad pituitary.

The neuro-intermediary lobe has a growth, thyrotropic, adrenotropic factor, and weight-increasing action on the spleen. These actions are much less pronounced than the corresponding actions of the anterior lobe. Thyrotropic and gonadotropic actions could not be demonstrated.

I wish to express my indebtedness to Professor B. A. Houssay whose advice and guidance made this work possible.

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48-HOUR ASSAY TEST FOR EQUINE GONADOTROPIN WITH RESULTS EXPRESSED IN INTERNATIONAL UNITS

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OUR PREVIOUS METHODS for assaying equine gonadotropin (1, 2) involved autopsy of the test rats 120 hours after injection. The primary purpose for this long test interval was to obtain information on the qualitative ovarian response in follicular growth, luteinization, and interstitial cell development. This information was considered essential because, conceivably, the gonadotropic activity of pregnant mare serum might depend upon two or more gonadotropic hormones. We therefore wished to compare the nature of the responses obtained from sera collected at different intervals during pregnancy as well as the responses of various fractions obtained during studies on methods of purification.

After comparing the qualitative and quantitative gonad response to extracts testing 4,000 to 7,000 R.U. per mg. total solids, with the response to untreated mare serum in normal and hypophysectomized male and female rats, we concluded that mare serum contains but one gonadotropic hormone (3). Three papers (4–7), give evidence in support of two or more gonadotropic hormones in the serum of pregnant mares. One is from this laboratory (4), but later it appeared (5) that the second fraction, inert by itself, consisted merely of serum proteins, since we obtained similar augmentation effects by combining other inert proteins such as casein or egg albumin with certain pituitary extracts. The second paper (6) was a preliminary note, not amplified thus far. Finally, Evans et al. (7) have reported three gonadotropic hormones in mare serum. This work has been neither confirmed nor disproved but to judge from the results (3) obtained from graded doses of purified extracts, these workers were observing differences in the qualitative response which were dependent upon the amount of the hormone administered. Unfortunately, they did not study the qualitative response over a series of dosage levels. Thus, although purifications of two thousand-fold or more have been effected, there is no satisfactory evidence that more than one gonadotropic hormone exists in PMS. Having reasonable assurance that only one gonadotropic hormone is present (3) in PMS, we have developed a shorter method of assay which is dependent only upon the quantitative response.

EXPERIMENTAL PROCEDURE

All rats used in this study were normal immature females 25 days old at the time of injection. The hormone, in all instances, was given in a single injection. To determine how much the test could be shortened, we injected graded doses of hormone and weighed the ovaries at 48, 72, 96, or 120 hours after injection (table 1). The differences between mean ovarian weights were less significant at 120 hours than at any other interval tested. In the dosage zone of 4 to 10 R.U., the difference between responses dependent upon dosage is as significant at 48 hours as it is at longer intervals.

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Since significant differences in ovarian weight were obtained after 48 hours, rats were autopsied at 24 hours after injection to determine whether the test period could be still further shortened. Table 2 shows the ovarian and uterine weight responses. As revealed by inspection of the data, this interval is too short for accurate assay, particularly since an actual decrease in ovarian weight may be obtained when the dosage is increased above 15 R.U.

TABLE 1. OVARIAN RESPONSE OF RATS INJECTED WITH EQUINE GONADOTROPIN AND AUTOPSIED AT VARIOUS INTERVALS

Dosages Compared	48 hours		72 hours		96 hours		120 hours	
	Mean ovarian weights at each dosage level	Value of P	Mean ovarian weights at each dosage level	Value of P	Mean ovarian weights at each dosage level	Value of P	Mean ovarian weights at each dosage level	Value o P
R.U.	mg.		mg.		mg.		mg.	
None, 4	16.8, 21.1	0.5<P<0.1	19.5, 32.1	<0.01	23.2, 25.0	0.4 <P<0.5	20.0, 24.1	0.2 <P<0.3
None, 6	16.8, 30.5	<0.01	19.5, 37.3	0.01<P<0.02	23.2, 28.8	0.05<P<0.1	20.0, 36.3	0.01<P<0.02
4, 6	21.1, 30.5	<0.01	32.1, 37.3	0.4 <P<0.5	25.0 28.8	>0.9	24.1, 36.3	0.05<P<0.1
4, 8	21.1, 38.4	<0.01	32.1, 49.2	<0.01	25.0, 46.3	<0.01	24.1, 41.0	0.02<P<0.05
6, 8	30.5, 38.4	0.1 <P<0.2	37.3, 49.2	0.1 <P<0.2	28.8, 46.3	<0.01	36.3, 41.0	0.6 <P<0.7
6, 10	30.5, 46.4	<0.01	37.3, 50.8	0.1 <P<0.2	28.8 61.0	<0.01	36.3, 47.8	0.3 <P<0.4
6, 12	30.5, 50.5	<0.01	37.3, 82.7	<0.01	28.8, 78.4	<0.01	36.3, 72.6	0.02<P<0.05
8, 10	38.4, 46.4	0.1 <P<0.2	49.2, 50.8	0.8 <P<0.9	46.3, 61.0	0.05<P<0.1	41.0, 47.8	0.6 <P<0.7
8, 12	38.4, 50.5	0.05<P<0.1	49.2, 82.7	<0.01	46.3, 78.4	<0.01	41.0, 72.6	0.05<P<0.1
10, 12	46.4, 50.5	0.4 <P<0.5	50.8, 82.7	0.02<P<0.05	61.0, 78.4	0.05<P<0.1	47.8, 72.6	0.2 <P<0.3

When P is 0.05 or less, the difference may be considered significant. Each mean is the average of the weights of 6 pairs of ovaries

TABLE 2. OVARIAN AND UTERINE RESPONSE TO EQUINE GONADOTROPIN OF RATS AUTOPSIED 24 HOURS AFTER A SINGLE INJECTION

Dose	No. of rats	Average weight of ovaries	Average weight of uteri
R.U.		mg.	mg.
None	11	13.3	17.5
2.5	8	16.3	28.6
5	8	17.8	31.8
10	8	29.7	36.0
15	7	30.8	35.6
20	7	20.3	35.1
40	8	21.8	29.9
80	8	25.1	35.2
160	8	25.8	36.4

Relation of rat unit to the international unit. We have determined the activity of the International Standard equine gonadotropin by 3 assay methods; (*a*) the smallest amount necessary to produce an average of 3 corpora or ripe follicles per rat (1); (*b*) the ovarian weight response at 120 hours (2); and, finally the ovarian weight response at 48 hours. By the first method, using 10 rats at each level, 0.125 mg. of International Standard gonadotropin produced an average of 1.4 corpora per rat; 0.1875 mg., an average of 2.8 corpora; 0.25 mg., the International Unit, an average of 6.4 corpora. Our rat unit, accordingly, is equivalent to the International Unit by this method. D'Amour and D'Amour (8) reported 44% estrous smears with 1 I.U. PMS, but no corpora. One hundred per cent of the rats in our experiments had estrous smears at this dosage level. The lack of corpora and the fewer positive smears found by D'Amour and D'Amour may be explained by the fact that they gave 3 injections of PMS instead of one as we did.

By the weight method, at 120 hours, 8 I.U. produced an average ovarian weight of 40.6 mg. in a group of 8 rats. This is just below the weight limits previously set for

8 R.U. (42 to 54 mg.) (2); thus, again, our rat unit is approximately equivalent to the international unit.[1]

Table 3 shows the ovarian response to the international standard at 48 hours, using 6 rats at each level. It also shows the response of an untreated serum (*serum 24*), the activity of which had been previously determined by the minimal response method (that is, the smallest amount necessary to produce an average of 3 corpora per rat). Here, again, our rat unit appears equivalent to the international unit. There is considerable error, however, in the test conducted by the minimal response method; less than a 50% increase in hormone content can not be detected. Thus, if the potency of a preparation is determined by the minimal response method, the ovarian weights resulting from dosages expressed in rat units may not necessarily conform so closely to ovarian weights resulting from the use of identical numbers of international units of the preparations (table 1).

TABLE 3. 48-HOUR OVARIAN RESPONSE TO INCREASING DOSAGES, EXPRESSED IN I.U., OF INTERNATIONAL STANDARD COMPARED WITH THE RESPONSE TO INCREASING DOSAGES, EXPRESSED IN R.U., OF AN UNTREATED SERUM (*Serum 24*). The significance of the differences between mean ovarian weights is also given.

International Standard			Serum 24		
Dosages compared	Mean ovarian weights at each dosage level	Value of P	Dosages compared	Mean ovarian weights at each dosage level	Value of P
I.U.	mg.		R.U.	mg.	
None, 2	13.7, 16.0	$0.02<P<0.05$			
None, 4	13.7, 19.3	$0.01<P<0.02$	None, 4	13.6, 19.3	<0.01
2, 4	16.0, 19.3	$0.02<P<0.05$			
4, 6	19.3, 26.3	<0.01	4, 6	19.3, 23.4	$0.01<P<0.02$
6, 9	26.3, 33.7	$0.01<P<0.02$	6, 8	23.4, 27.3	$0.02<P<0.05$
6, 13	26.3, 34.3	<0.01	6, 12	23.4, 34.5	<0.01
9, 13	33.7, 34.3	$0.7<P<0.8$	8, 12	27.3, 34.5	$0.02<P<0.05$
13, 20	34.3, 42.7	<0.01	12, 16	34.5, 39.5	$0.3<P<0.4$
20, 30	42.7, 50.2	$0.05<P<0.1$	16, 24	39.5, 47.5	$0.1<P<0.2$
30, 45	50.2, 53.7	$0.4<P<0.5$	24, 40	47.5, 55.4	$0.1<P<0.2$

When P is 0.05 or less, the difference may be considered as significant. Each mean is the average of six pairs of ovaries.

Are uterine or ovarian weights more reliable? In the work of Levin and Tyndale (9), uterine weights of mice afforded a more sensitive means of determining the gonadotropic potency of castrate or post-menopausal human urines than ovarian weights did. Some preliminary observations led these workers to consider the method less accurate for pregnant mare serum. According to D'Amour and D'Amour (8), however, the uterine weight-vaginal smear technic appears most satisfactory for equine gonadotropin. Evans and his co-workers (10) found the uterus more sensitive than the ovary to PMS. Hamburger and Pedersen-Bjergaard (11) decided that PMS could best be standardized by the ovarian response. Uterine weights were obtained in the group of animals upon which table 1 is based. The uterine horns were severed at the point of bifurcation, and the fluid was drained from the uterine cavity before weighing. In this group of rats we found less significance in the differences between uterine weights dependent upon dosage than for the ovarian weights. Because the first steep rise in uterine

[1] One sample of International Standard gonadotropin tested lower than this by both of the methods mentioned above (1 R.U. was equivalent to 1.5 to 2 I.U.); but as this particular sample was stored at room temperature for some time, the possibility of deterioration must be considered. Furthermore, the weight of this particular sample was not checked. Preliminary observations on the activity of this sample were the basis for indicating (3) that our rat unit was equivalent to 2 I.U. Because of the uncertainty associated with it, we are ignoring the results on this sample.

weights occurred at lower dosage levels than those used in this series, another series of experiments was run in which the response to doses of 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, and 2.0 R.U. of gonadotropin were compared, 8 rats being used at each dosage level. In this series the uteri were severed at the junction with the cervix, and the fluid was not drained from the uterine cavities. Although the uterine weights ranged from an average of 29.8 mg. for the controls to 120.9 mg. for those receiving 2 R.U., the individual weight variations made the differences less significant than was true for ovarian weights at higher dosage levels used in the series shown in table 1. The average uterine weights, with the standard errors for the differences between means, are shown in table 4. We conclude, therefore, that in testing for equine gonadotropin the weight of the rat ovary is more accurate, though less sensitive, than the weight of the rat uterus.

TABLE 4. 48-HOUR UTERINE RESPONSE TO INCREASING DOSAGES OF UNTREATED PMS (*Serum 24*) WITH THE SIGNIFICANCE OF THE DIFFERENCES BETWEEN MEAN UTERINE WEIGHTS.

Dosages compared	Mean uterine weights at each dosage level	Value of P
R.U.	mg.	
None, 0.6	29.8, 57.1	$0.01<P<0.02$
None, 0.8	29.8, 61.1	<0.01
0.6, 0.8	57.1, 61.1	>0.9
0.6, 1.0	57.1, 86.7	$0.05<P<0.1$
0.6, 1.2	57.1, 107.7	<0.01
0.8, 1.0	61.1, 86.7	$0.05<P<0.1$
0.8, 1.2	61.1, 107.7	<0.01
0.8, 1.4	61.1, 116.7	<0.01
0.8, 1.6	61.1, 129.8	<0.01
1.0, 1.2	86.7, 107.7	$0.1<P<0.2$
1.0, 1.4	86.7, 116.7	$0.05<P<0.1$
1.0, 1.6	86.7, 129.8	$0.01<P<0.02$
1.0, 2.0	86.7, 120.9	<0.01
1.2, 1.4	107.7, 116.7	$0.5<P<0.6$
1.2, 1.6	107.7, 129.8	$0.1<P<0.2$
1.2, 2.0	107.7, 120.9	$0.1<P<0.2$
1.4, 1.6	116.7, 129.8	$0.4<P<0.5$
1.4, 2.0	116.7, 120.9	$0.7<P<0.8$
1.6, 2.0	129.8, 120.9	$0.5<P<0.6$

When P is 0.05 or less the difference may be considered significant. Each mean is the average of 8 uteri.

The 48-hour assay method using ovarian weights. As a basis for assay the data obtained on the International Standard (table 3) is used. Unfortunately, there was not enough of this material to include more than 6 rats at a given dosage level. Figure 1 represents the regression line calculated by the method of least squares on the basis of average ovarian weights obtained by administering 4, 6, 9, 13, and 20 I.U. of International Standard gonadotropin. Values obtained by dosages of less than 4 I.U. or more than 20 I.U. were not used since previous experiments had indicated that only over this range might one expect a straight line in plotting ovarian weight against dosage. The standard error of estimate is also included, together with the values actually observed for International Standard and for one other gonadotropic preparation, *serum 24*. The observed values for *serum 24* fit fairly closely the regression line calculated on the basis of values obtained on the International Standard. Using this line one can determine the potency of gonadotropic preparations by interpolation on the basis of average ovarian weight if the ovarian weight of a group of rats receiving a given dosage of a gonadotropic preparation falls between 25 and 40 mg. Still greater accuracy might be obtained by administering, to different groups of rats, varying

dosages of such magnitudes that the ovarian weights fall on several points of the line between 25 and 40 mg. and averaging the values obtained by interpolation.

With a constant standard error of estimate, irrespective of the ovarian weight range, the relative accuracy would appear to be greater for ovarian weights falling between 35 and 40 mg. than between 25 and 30 mg. To test this point the standard deviation of I.U. values, obtained by interpolation of individual ovarian weights using the regression line, was determined from the actual number of I.U. administered. Five groups in which the average ovarian weight was between 25 and 30 mg. and 8 groups

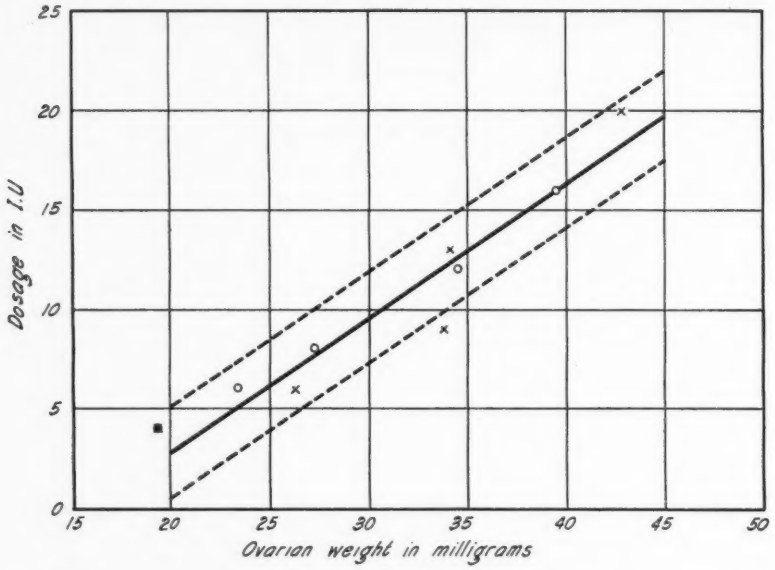

Fig. 1. Regression line calculated by the method of least squares on the basis of average ovarian weights obtained by administering 4, 6, 9, 13, and 20 I.U. of international standard with 6 rats at each dosage level. The standard error of estimate, ±2.24 I.U., is indicated by the dotted lines. The actual values upon which the line is based are indicated by crosses; the circles represent values obtained on *serum 24*, assuming that the rat unit is equivalent to the international unit.

in which the average ovarian weight was between 35 and 40 mg. were analyzed. The International Standard was not used for all groups. Where other gonadotropic preparations were used, the number of I.U. was determined by interpolation of average ovarian weights. A group consisted of 6 to 8 rats receiving a constant amount of a given gonadotropic preparation. The average standard deviation was ±1.42 I.U. for the groups falling between 25 and 30 mg. ovarian weight. ±1.82 I.U. for the groups falling between 35 and 40 mg. ovarian weight. The difference between the average standard deviations is small, and is not statistically significant. Conceivably, therefore, greater relative accuracy is possible in the higher range of ovarian weight.[2]

SUMMARY

Since earlier work has indicated that only one gonadotropin exists in pregnant mare serum, a method of assay based solely upon a quantitative ovarian response has

[2] We wish to express our appreciation to Dr. Max Kleiber for advice on the statistical analysis of the data.

been devised. The ovarian weight response to equine gonadotropin at varying dosage levels was determined at 24, 48, 72, 96, and 120 hours after a single injection. The differences between mean ovarian weights, dependent upon dosage, were as significant at 48 hours as they were at longer intervals. Our rat unit, used for several years, is approximately equivalent to the International Unit. Contrary to the findings of others using human urine gonadotropins, it appears that the ovarian response to equine gonadotropin is more reliable than the uterine response and the ovarian weight method has the added advantage of being a direct response. Using values obtained by use of the International Standard, a regression line that may serve for determining potency of equine gonadotropins by interpolation has been calculated.

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THE REACTION OF HUMAN CHORIONIC AND EQUINE GONADOTROPINS TO FORMALDEHYDE

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SINCE THE VARIOUS GONADOTROPINS PRODUCE well-defined differences in physiologic response, differences in physical properties and chemical structure have been sought. Several attempts purporting to show a chemical difference (1, 2) have been refuted (3, 4, 5). Li, Simpson, and Evans (6) and Li, Evans, and Wonder (7) reported that the inactivation by ketene of the physiologic activity of pituitary and mare serum gonadotropins is more rapid than the inactivation of human chorionic gonadotropin. To them the results[1] suggest that free amino groups are essential for the biological activity of the pituitary and mare serum hormones. In this respect these hormones are to be contrasted with human chorionic gonadotropin. The observation of Cartland and Nelson[2] (8) that 84% of the mare serum hormone was destroyed by 4% formalin in 3 hours at pH 8.0 is cited as confirmatory evidence. It is known, Li et al. state, that formaldehyde reacts only with the free amino groups under the cited conditions. Studies on insulin (9–13) have shown rather conclusively that formaldehyde reacts with other groups under conditions of the formol titration. Moreover, the reaction in the formol titration is rapid and under conditions in which the free amino group has reacted in the formol titration, considerable activity of sheep pituitary, equine, and human chorionic gonadotropins has been recovered (14, 15, 16).

It is the purpose of this paper to compare the inactivation by formaldehyde of mare serum hormone and chorionic gonadotropin (prolan) under identical conditions of formaldehyde concentration, pH, and reaction time, in an effort to detect a difference in chemical structure. A concentration of 4% formaldehyde in disodium phosphate or sodium bicarbonate buffer solution was used as the reaction medium. The reaction was terminated either by dilution and immediate dosage, or by conversion of the formaldehyde to hexamethylene tetramine with an excess of ammonia. At the pH employed hexamethylene tetramine does not liberate formaldehyde (17).

The possibility still exists that the prosthetic groups of all the gonadotropins partake of the same general structure, any difference in physiologic response being the result of differences in physical properties of the molecule or micelle as a whole which

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[1] Their data reveal unusual results for the prolan experiments, in that no inactivation results for 15 minutes and then 83% inactivation results in the next 30 minutes. The other hormones are inactivated 90 to 97% in 30 minutes. It is stated that in all experiments except the prolan experiment the reaction solution contained 2 mg. protein per cc. The prolan was in a concentration of 10 mg. per cc. We suggest that the delay for prolan inactivation may possibly be due to initial destruction of ketene by impurities.

[2] The experimental conditions chosen by Cartland and Nelson (8) were the same as those used by us (14) in studying the action of formaldehyde on the pituitary hormone. These conditions were not designed to study reaction time, as a vacuum distillation follows the main reaction, nor was quantitative recovery assured by the subsequent alcohol precipitation. They were rather designed, as Cartland and Nelson used them, to ascertain whether a selective inactivation of one of two hypothetical gonadotropic hormones was effected.

might affect the rate of resorption, permeability, destruction, excretion, or stimulation to antibody formation. In any chemical reaction study it is therefore important to ascertain whether an apparent inactivation is not in reality due to a change in resorption or stability which would be revealed, as we have previously shown (3, 5) if a comparison between two assay procedures employing different resorption rates (divided versus single doses) is made. This criterion has again been employed in the present studies.

EXPERIMENTAL

Assay of reaction products. Ovarian weight increase served as the objective measure (16) in the assay of the mare serum hormone. In all experiments, 22 to 23-day-old female rats were used to assay the reaction product, equal numbers of littermates being used for a simultaneous assay for an aliquot of material which had not been subjected to formaldehyde reaction. In some cases the control solution was assayed at two dosage levels. In those experiments in which the reaction was terminated by dilution and immediate dosage, one dose per rat was given. In those experiments in which the reaction was terminated by conversion of formaldehyde to hexamethylene tetramine four doses were given. Ovarian response to mare serum hormone is very nearly the same whether single or multiple doses are made (18). A reaction product of formaldehyde and ammonia was added to the control solution in both mare serum and prolan experiments in an amount corresponding to that formed in the experimental procedure.

In the case of prolan two assay procedures one of which depended on ovarian weight increase, the other on uterine weight increase, were used as a check against each other. In those experiments in which formaldehyde was converted to hexamethylene tetramine at the end of the reaction period, the uterine assay procedure of Delfs (19) was used; in the other experiments that of D'Amour (20) was used, it being necessary to repeat the chemical reaction 3 times because of the dosage schedule. The relation between ovarian weight and hormone level in our assay procedure may be gauged from the following data (24, 18, 18 or 12 rats per level): 0.5 mg., 0.4 mg., 0.25 mg., and 0.20 mg. of prolan produced respectively 36 mg., 32 mg., 27 mg., and 25 mg. ovaries. In assays in which 6 and 7 groups of littermates were used, a 20% increment (20% of the largest dose) in dose level corresponded to 1.9 times the standard deviation of the mean, a 30% increment corresponded to 2.8 times and a 50% increment corresponded respectively to 4.0 and 4.8 times the standard deviation of the mean. The ovarian weight mean would deviate not more than 25% from the correct assay value once in 25 to 50 times.

Six to 8 pairs or trios of litter mates were used per assay based on ovarian weight and 11 pairs or trios were used for the uterine weight procedures. The % recovery of hormone was calculated from the estimated units of the control and experimental doses obtained from the standard reference curves on the basis of the organ weights observed.

A control for 0 time in the experiments in which formaldehyde was not converted to hexamethylene tetramine was made by adding the hormone to a buffered formaldehyde solution just before dosage and comparing the result with a formaldehyde-free solution. The concentration of formaldehyde was the same as that resulting after dilution in the inactivation experiments. Results for mare serum hormone were: 67 ± 2.8 mg. versus 67 ± 2.5 mg. ovaries, 7 pairs of litter mates. Results for prolan were: 38 ± 1.6 mg. versus 38 ± 3.2 mg. ovaries, 7 pairs of litter mates.

Assay pitfalls. The assay of the mare serum hormone presents no difficulties. A difference of opinion exists as to the best assay procedure for prolan. The deductions

made in this study are dependent upon the accuracy of the prolan assay, and for this reason two procedures were chosen. The uterine test is an indirect test depending upon the ability of prolan to stimulate the ovary to secretion of ovarian hormones. It is theoretically possible that chemical treatment might alter the prolan so that its gonadotropic powers were not altered, but its powers to stimulate ovarian secretion were altered. The results of the 2 assay procedures would, therefore, not necessarily be in agreement and a statistically significant difference would indicate that the physiologic properties had been altered. Within the error of assay, no such change is indicated in the present experiments.

A number of workers (19) have stated that the uterine response for prolan is a

TABLE 1. INACTIVATION OF PROLAN AND EQUINE GONADOTROPIN BY 4% FORMALDEHYDE AT pH 8.5

Exp. no. and conditions prior to dosage	Reaction time in hours	Ratio: control dose / exp. dose	Mean[3] organ wt. for control dose	Mean organ wt. for exp. dose	Estimated % recovery of hormone
			Equine Gonadotropin		
1, A[1]	0.25	1.0	62±2.5 (o)[4]	55±2.3 (o)	90
2, A	1	0.5	40±2.8 (o)	41±2.8 (o)	50
3, B[2]	1	0.56	42±2.6 (o)	31±1.1 (o)	45
4, A	3	0.25	36±2.2 (o)	64±3.7 (o)	35
5, B	3	0.40	36±2.7 (o)	30±3.1 (o)	30
		0.20	25±2.0 (o)	30±3.1 (o)	
			Prolan		
6, A	1	1.0	95±4 (u)[5]	63±9 (u)	45
7, B	1	0.8	35±2.2 (o)	32±1.2 (o)	65
8, B	1	0.5	70±8 (u)	53±10 (u)	45
		1.0	85±5 (u)	53±10 (u)	
9, A	3	0.8	30±1.6 (o)	21±1.5 (o)	40
		0.6	26±1.4 (o)	21±1.5 (o)	
		0.8	33±1.2 (o)	25±1.2 (o)	
		0.4	24±1.4 (o)	25±1.2 (o)	
10, A	3	1.0	92±6 (u)	21±1.5 (u)	35
11, B	3	0.8	33±1.2 (o)	26±1.2 (o)	50
		0.4	24±1.4 (o)	26±1.2 (o)	
12, B	3	0.5	70±8 (u)	20±1.6 (u)	<35
		1.0	85±5 (u)	20±1.6 (u)	
13, B	6	0.4	27±1.1 (o)	25±1.4 (o)	30

[1] A = 10 to 50 I. S. U of prolan or 25 to 200 Cole U mare serum hormone with 10 mg. $NaHCO_3$ or $Na_2HPO_4 \cdot 2\,H_2O$ per cc.; formalin pH 8.5 added to make 4% formaldehyde concentration; appropriate dilution after reaction; immediate dosage.

[2] B = same reagents as A; after reaction, cooling in ice; 2.6 moles NH_3 for each mole HCHO, HOAc to take pH to 8.5.

[3] Mean and standard deviation of mean.

[4] o = assay performed by ascertaining ovarian weight increase.

[5] u = assay performed by ascertaining uterine weight increase.

more satisfactory end point because of greater sensitivity than is increase in ovarian weight. An examination of the data of these workers reveals that in the ovarian weight range used, which is the least sensitive range, a 10 mg. increase in ovarian weight (15 mg. to 25 mg. ovaries) corresponds to a 4 to 15-fold increase in dosage. At the ovarian weight range employed in our assay (25 mg. to 35 mg. ovaries) a 10 mg. increase in ovarian weight is produced by doubling the dose. In our experience with the uterine assay procedures, the marked increase in uterine weight which is brought about by a small increment in dosage (25%) is accompanied by considerable variation in response, which results in a high standard deviation of the mean. Our data reveal that the ovarian assay procedure is as accurate as the uterine assay procedure, con-

firming the experience of Sealy and Sondern (19). Ovarian weight assay should not be condemned on the basis of observations which compare the range of greatest sensitivity for the uterine assay with the range of least sensitivity for the ovarian weight assay, or which employ a dosage schedule which does not bring about maximal ovarian response.

Data. Details of the reaction conditions, the number of litter mates used per assay and the results of the assay are given in table 1. Phenolphthalein in a concentration of 1 mg. per 5 cc. was used to control pH. The reaction in formaldehyde took place at room temperature. The prolan was the same as that used in earlier studies (15). A number of batches of Cutter's gonadin, assaying 2,000 U. per mg. nitrogen, were used.

RESULTS

The results of *experiment 1* show that when mare serum gonadotropin is subjected to 4% formaldehyde at pH 8.5 for 15 minutes at room temperature 90% of the activity is recovered. The extension of the reaction to 1 hour resulted in 50 and 55% inactivation (*experiments 2* and 3). An exposure of 3 hours produced 65 and 70% inactivation in *experiments 4* and 5. In *experiments 3* and 5, formaldehyde was converted to hexamethylene tetramine at the end of the reaction period. The results confirm the experiments (2 and 4) in which the reaction was terminated by dilution and immediate dosage.

The results of the prolan experiments for 1 and 3 hours exposure to formaldehyde (*experiments 6–12*) match those for the equine gonadotropin experiments within the error of assay. The assay values for prolan obtained by uterine weight are nearly identical with the corresponding values for the mare serum determination: at 1 hour, 45 and 45% recovery versus 50 and 45%; at 3 hours, 35 and 35% recovery versus 30 and 35%.

In the case of the mare serum experiments the assays performed with 1 dose (*experiments 2* and 4) confirm those (*experiments 3* and 5) performed with 4 doses. Likewise in the prolan experiments, the assays performed by the method of Delfs (*experiments 8* and *12*) employing 3 doses per day confirm the assays performed by the method of D'Amour and D'Amour (*experiments 6* and *10*), which employ 1 dose per day. It would, therefore, appear that the partial inactivation of the gonadotropins by formaldehyde is not apparent, due to a change in rate of resorption or destruction by the body, but actually is a change in the prosthetic group.

Attempts at reactivation. An attempt to reactivate the prolan formaldehyde reaction product by heating 15 minutes at 37° C., pH 6.0, failed (28 rats in assay). Likewise an attempt to reactivate the mare serum formaldehyde product by exposure to pH 2.0 for 1 hour at room temperature failed (16 rats in assay).

DISCUSSION

The foregoing experiments offer no evidence that the free amino group is essential to mare serum hormone activity and non-essential to prolan activity. They do indicate, however, that some other reaction not involving the amino group takes place between formaldehyde and the hormones, producing gradual partial inactivation. This reaction may be akin to that produced by cysteine and cyanide (5), which is also a comparatively slow inactivation. The recovery of 30% of the activity of both hormones after 3 hours exposure to formaldehyde indicates: *a*) that the free amino group is not essential for the activity of the hormones; *b*) that the amino groups do not completely react; *c*) that the methyleneated amino compound is physiologically less active; *d*) that the reaction involving an amino group is partially reversible. The second explanation is the least likely, in view of the behavior of other proteins to formalde-

hyde action in which the reaction is completed in a few minutes. The last explanation is also unlikely since in the case of collagen, the reaction with formaldehyde in neutral or alkaline solution has been shown to be irreversible (21), and since attempts to produce reactivation in our experiments failed.

SUMMARY

The reaction of mare serum hormone or prolan in 4% formaldehyde concentration at pH 8.5 was terminated either by dilution of the reaction product and immediate dosage or by conversion of the excess formaldehyde to hexamethylene tetramine by ammonia.

One and 3 hours exposure to formaldehyde produced progressive partial inactivation of both hormones and within the limitations of the assay to the same degree.

One quarter-hour exposure of mare serum hormone to formaldehyde resulted in almost complete recovery of active material.

Under the conditions of the formaldehyde reaction in these experiments no difference in the chemical structures of mare serum hormone and prolan could be detected. There is no indication that the free amino group is essential to mare serum activity and non-essential to prolan activity.

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SELECTIVE NEUTRALIZATION OF THE FOLLICLE-STIMULATING ACTION OF GONADOTROPIC PREPARATIONS BY ANTIGONADOTROPIC SERA[1]

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NUMEROUS INVESTIGATORS HAVE SHOWN that antigonadotropic substances can be produced in animals injected for long periods of time with gonadotropic extracts. The reader is referred to a review paper by Collip, Selye and Thomson (1) for a discussion of the literature on this subject. The only attempts to produce an antisubstance specific for one component of the gonadotropic hormone of the anterior pituitary gland was made by Rowlands (2, 3). He reported the selective neutralization of the luteinizing hormone (LH) of beef pituitary gland by antisera developed in rabbits which had been injected repeatedly with beef pituitary extracts. These antisera, however, evidently contained some antifollicle stimulating hormone action (anti-FSH). The data he presented showed that upon administration of quantities of the serum sufficient to prevent luteinization there was a small increase in the weight of the ovaries of immature female rats receiving both antiserum and horse pituitary extract, indicating that inhibition of the follicle-stimulating activity of the extract had occurred. In view of these findings it was of interest, therefore, to determine whether an antisubstance specific for the follicle-stimulating hormone of the gonadotropic complex could be produced in rabbits by the repeated injections of FSH preparations. Characterization of the anti-FSH serum was determined by the following experiments: (*a*), injection of anti-FSH and FSH into the immature female rat to establish the presence of an inhibitory substance for FSH in the antiserum; (*b*), administration of anti-FSH and LH to immature male rats to show the absence of anti-LH action in the serum; and (*c*), simultaneous injection of anti-FSH and unfractionated gonadotropic extracts into both immature male and female rats to demonstrate that anti-FSH serum is capable of neutralizing, selectively, the FSH activity of unfractionated extracts.

PROCEDURE

The FSH preparation used in this study for the production of the anti-FSH serum was prepared by tryptic digestion of sheep pituitary extracts according to the method of McShan and Meyer (4) and was assayed in 21-day-old female rats of the Sprague-Dawley strain. A dose of 0.5 gm. equivalent of dried gland was given to each of the assay animals in 9 injections of 0.5 cc. each for 4.5 days. The animals were autopsied on the morning of the sixth day, at which time it was found that the uteri were distended with fluid. The ovaries had an average weight of 47 mg. and contained well developed follicles but no corpora lutea. In addition to the FSH extract an unfrac-

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tionated aqueous extract of sheep pituitary glands was used for the preparation of an antigonadotropic serum (anti-SAP) for the whole gland. This extract when given to 21-day-old rats in 9 injections during 4.5 days produced ovaries which had an average weight of 75 mg. and which contained many corpora lutea.

Antisera which would prevent the gonad-stimulating action of FSH preparations or of unfractionated extracts of whole dried sheep pituitary glands, were prepared by injecting one group of female rabbits with FSH extracts and a second group with unfractionated extracts of whole sheep glands. The extracts were prepared so that 1 cc. contained activity equivalent to 250 mg. of whole dried pituitary gland. One cc. of each of the preparations was injected subcutaneously each day for a period of 2 months. After 2 to 3 months of injections, blood was obtained from the rabbits by cardiac puncture and serum prepared by centrifugation. Two pooled lots of anti-FSH serum and one pooled lot of anti-SAP serum were made. These will be referred to as anti-FSH I and II and anti-SAP, respectively.

Tests for the antigonadotropic properties of the serum were made by injecting 21-day-old female rats with either 1 cc. of anti-FSH II or anti-SAP, or 2 cc. of anti-FSH I with the extract used in their preparation and also with purified pregnant mare serum (PMS). The antiserum and the gonadotropins were injected simultaneously but each into different parts of the body. Nine injections of each were given during a period of 4.5 days and the animals were autopsied on the morning of the sixth day.

Although it was found that the three lots of rabbit serum contained a substance which neutralized the action of FSH, it was desirable to determine whether or not they had the power to inhibit the action of LH. The presence of any anti-LH activity in the sera, however, could not be detected by the use of the immature female rat since the antigonadotropic activity of the serum would inhibit the follicle-stimulating action of the extracts and hence there would be no growing follicles upon which the LH could act to produce corpora lutea. To meet this difficulty immature male rats were used to determine whether or not the sera obtained from the two groups of rabbits injected with FSH and unfractionated gonadotropic hormone of sheep hypophysis contained any anti-LH action. In the immature male rat FSH is mainly gametogenic. The LH, however, stimulates only the interstitial cells, causing an increase in the production of male hormone which produces an increase in the size of the male accessories[2] (5). It is this secondary effect on the seminal vesicles and prostate with which we are especially concerned. If the serum of the rabbits injected with FSH is specific for the FSH, it would not inhibit the action of the LH of simultaneously administered gonadotropins. To test this theory the antisera which showed an inhibitory action for FSH in the immature female rat were injected into immature male rats together with three types of gonadotropic hormones—LH, SAP and PMS. The same procedure which has been used for testing the inhibitory action of antisera in female rats was also used in testing for the antigonadotropic properties of the sera in male rats with the exception that 2 cc. of the anti-FSH sera and 1 cc. of the anti-SAP serum was administered

RESULTS AND DISCUSSION

The antigonadotropic activity of the anti-FSH I serum was determined first by the ability of the serum to inhibit the gonad-stimulating action of FSH in the immature female rat. The average ovarian weight obtained in 12 immature female rats receiving injections of both FSH and anti-FSH I was 13 mg., there being no increase

[2] The term 'male accessories' used in this report refers to the seminal vesicles and prostate, the prostate including the coagulating gland.

in weight over that found in the uninjected controls. Follicle-stimulating preparations, however, when given to 14 twenty-one-day-old female rats for a period of 4.5 days caused the weight of the ovaries to increase from the normal value of 13 mg. to an average weight of 75 mg.

To determine whether or not there was any anti-LH activity present in the serum, the anti-FSH serum was injected with LH into the male rat. The data in table 1 show that no inhibition of the LH activity was obtained by the use of the anti-FSH serum since the response of the male accessories was the same in the rats simultaneously injected with LH and anti-FSH as was that obtained in the male rats injected with LH alone. Other data are given in table 1 which show the effect of injecting an antigonadotropic substance, which was active against unfractionated SAP, together with LH on the gonads and accessories of male rats. In these animals the average weights of the seminal vesicles and prostate of the rats receiving the combined injections of

TABLE 1. INHIBITORY ACTION OF ANTI-FSH AND ANTI-SAP ON THE EFFECT OF LH IN THE IMMATURE MALE RAT

No. of rats	Treatment and Dose	Testes	Seminal Vesicle	Prostate
		mg.	*mg.*	*mg.*
12	Anti-FSH 2 cc. LH 1 gm.[1]	411	17.5	82.3
10	LH 1 gm.[1]	442	16.5	71.2
9	Anti-SAP 1 cc. LH 1 gm.[1]	244	7.0	34.0
17	Controls, no injections	304	7.1	42.0

[1] In equivalents of dried pituitary powder.

LH and anti-SAP were definitely less than those of the accessories of the animals injected only with the LH, indicating that anti-SAP is effective in inhibiting completely the activity of LH. From these results we can conclude that although the anti-FSH serum is capable of inhibiting FSH as demonstrated in the female rat, it is ineffective against the action of LH as tested in the male rat, demonstrating an absence of anti-LH in the anti-FSH serum. The anti-SAP serum, in contrast, inhibited both SAP in the female rat and LH in the male rat, showing that the LH activity can be inhibited in the male rat by an antiserum produced by injecting an unfractionated sheep pituitary extract into rabbits.

In an attempt to determine whether or not anti-FSH serum could selectively neutralize the follicle-stimulating activity of unfractionated gonadotropic extracts without inhibiting the action of the luteinizing factor, the anti-FSH serum was injected with SAP and PMS into both immature male and female rats. The data for this experiment are presented in table 2. In addition to the two lots of anti-FSH sera, the anti-SAP serum was also injected with SAP and PMS to determine whether or not the follicle-stimulating and luteinizing action of these preparations could be prevented in male and female rats by the anti-SAP serum. The inhibitory effects of anti-FSH I and II and anti-SAP on the gonadotropic extracts were first demonstrated in the female rat. Two cc. of anti-FSH I or 1 cc. of anti-FSH II was sufficient to prevent the ovarian growth produced by FSH, SAP and PMS in the female rat, indicating that a suppression of the follicle-stimulating action of these extracts had occurred. Increasing the dose of anti-FSH II to 2 cc. and maintaining the same dose of anti-FSH I in

the male rat failed to demonstrate any antiluteinizing activity in these sera. The seminal vesicle and prostate weights of the male rats given anti-FSH I or II and gonad stimulating extracts were essentially the same as those of the animals injected with the gonadotropic extracts alone. Inasmuch as the LH causes growth of the male accessories, it is apparent that there is no neutralization of the luteinizing activity of the gonad-stimulating extracts by the anti-FSH sera, indicating a specificity of the anti-

TABLE 2. SELECTIVE NEUTRALIZATION OF THE FOLLICLE-STIMULATING ACTIVITY OF GONADOTROPIC PREPARATIONS BY ANTI-FSH SERA

Treatment	Female			Male				
	Dose	No. of rats	Ovaries	Dose	No. of Rats	Testes	Sem. Ves.	Prostate
			mg.			*mg.*	*mg.*	*mg.*
Anti-FSH I and PMS	2 cc. 0.5 mg.	5	25	2 cc. 0.5 mg.	5	399	33	111
Anti-FSH II and PMS	1 cc. 0.5 mg.	6	14	2 cc. 0.5 mg.	5	347	29	108
Anti-SAP and PMS	1 cc. 0.5 mg.	5	9	1 cc. 0.5 mg.	5	138	5	20
PMS[1]	0.5 mg.	10	194	0.5 mg.	8	527	33	112
Anti-FSH I and SAP	2 cc. 100 mg.	5	22	2 cc. 100 mg.	5	489	20	103
Anti-FSH II and SAP	1 cc. 100 mg.	6	14	2 cc. 100 mg.	5	404	21	102
Anti-SAP and SAP	1 cc. 100 mg.	5	11	1 cc. 100 mg.	5	175	6	27
SAP[2]	100 mg.	12	75	100 mg.	7	527	22	107
Uninjected Controls	—	7	13	—	8	408	7.5	44
Anti-FSH I and FSH	2 cc. 0.5 gm.	5	13					
Anti-FSH II and FSH	1 cc. 0.5 gm.	5	8					
FSH[2]	0.5 gm.	5	77					

[1] The PMS was a purified preparation made by the method of Cartland and Nelson (7).
[2] The quantities of these preparations injected are given in equivalents of dried pituitary powder.

FSH sera for FSH only. The selective neutralization of the FSH by anti-FSH sera without influencing the luteinizing activity of the gonadotropic extract is especially indicated in the case of anti-FSH II, when twice the quantity of serum necessary to produce inhibition of unfractionated gonadotropic extracts in female rats was injected into the male rat without any material effect upon the accessories. However, an increase in the size of the testes was prevented. Growth of the seminal vesicles and prostate with a diminution in the size of the testes in the rats injected with both anti-FSH sera and gonadotropic extract, shows that the antisera inhibited only the stimulating action of the extract on the seminiferous tubules (table 2). The interstitial

cells responsible for the growth of the accessories were not prevented from responding to the luteinizing activity of the gonad-stimulating preparations by the anti-FSH sera.

One cc. of anti-SAP serum inhibited the gonadotropic activity of SAP and PMS in both the immature male and female rat (table 2). Since this antiserum prevented the action of these two preparations on the gonads and accessories of the male it seems evident that neutralization of the effect of both FSH and LH had occurred.

In addition to the inhibitory action of anti-SAP serum on the gonadotropic hormone, the serum evidently suppressed the gonadotropic secretion of the pituitary gland of the assay animal. This statement is warranted by the fact that the testes and accessories of the rats receiving both anti-SAP and gonad-stimulating extracts were not only smaller than those receiving the extracts alone but they weighed less than those of the uninjected controls (table 2). Anti-FSH sera, however, did not cause a decrease in the size of these organs below that found in the uninjected males. These data suggest that an antiserum which prevents the action of the unfractionated gonadotropic complex is more efficient in neutralizing the action of exogenous and endogenous hormone than an antiserum active against one component of the complex.

The data which have just been discussed show that anti-FSH sera obtained from rabbits repeatedly injected with FSH preparations made by the tryptic digestion method selectively neutralized the follicle stimulating activity but not the LH activity of gonadotropic extracts. Serum from rabbits receiving equivalent doses of an undigested aqueous sheep pituitary extract failed to exhibit this specificity and inhibited both the follicle-stimulating and luteinizing effects of the gonadotropic preparations tested. These results provide evidence for the concept that extracts of PMS and SAP contain as separate entities a follicle-stimulating and a luteinizing hormone. However, another interpretation is suggested which has as its basis the idea that both the follicle-stimulating and luteinizing effects could be produced by a single gonadotropic complex. It also seems conceivable that an antiserum developed against an artificially produced component of this complex, such as is obtained by tryptic digestion, prevents the action of one part of the complex and leaves the other free to act.

In previous reports we have discussed the results obtained by the injection of an aspecific antigonadotropic sera into normal rats (5, 6). These results together with those given in the present report demonstrate the value of using antigonadotropic sera as a tool in the study of pituitary-gonadal interrelationships. It also seems probable that antisera developed against other anterior pituitary hormones would be useful for obtaining data concerned with the interrelationships between the anterior pituitary and other endocrine glands, particularly the thyroid.

SUMMARY

The serum of rabbits injected with an FSH preparation, made according to the tryptic digestion method of McShan and Meyer, selectively neutralized the follicle-stimulating activity of gonadotropic extracts. The anti-FSH activity of the serum was first demonstrated by the ability of the serum to inhibit the gonad-stimulating activity of FSH, PMS and SAP in the immature female rat. The absence of anti-LH properties in the serum was shown by its inability to prevent the increase in size of the seminal vesicles and prostate when injected simultaneously with LH into immature male rats. The anti-FSH serum selectively neutralized the FSH action of unfractionated extracts of SAP and PMS when the serum and either one of the extracts were administered to immature male rats. The luteinizing activity of these extracts, as determined by the response of the male accessories, was not neutralized by the anti-FSH serum. The serum from rabbits which received an unfractionated extract of

sheep pituitary glands inhibited the gonadotropic action of LH, SAP and PMS in both immature male and female rats.

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SPECIFICITY OF PREGNANCY URINE ANTIPROLAN WITH RESPECT TO HUMAN GONADOTROPIC HORMONES FROM VARIOUS SOURCES[1]

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IN 1937 WE INVESTIGATED QUANTITATIVELY the antigonadotropic activity of antiprolan towards gonadotropic hormones of various sources (1). The results showed that pregnancy urine prolan antiserum had less than 0.5% activity against the gonadotropin of animal pituitaries or pregnant mare blood. There was, however, a somewhat higher effectiveness against gonadotropic hormone of the same species, namely human pregnancy blood or human hypophysis, amounting to 7% of the antiprolan activity. Recently we have extended these studies to placental prolan and have also found a 7% activity of the antiserum, care having been taken to free the placenta thoroughly from blood remnants in order to assay pure placental prolan only.

The question then arose as to whether the low potency (7%) of the PU prolan antiserum against human gonadotropic hormone of other sources was due to the difference in origin or to a difference in hormone structure. As is well known, pituitary gonadotropin differs considerably from PU prolan in its content of Evan's synergist. It was of interest to investigate the behavior of PU antiprolan towards urine gonadotropin of pituitary origin, which is to be found in the urine of normal males and of women in the menopause (2).

Three different preparations were used for this assay: *a*) gonadotropic hormone from the urine of normal males (0.5 mg. = 1 R.U.)[2] *b*) gonadotropic hormone from the urine of women in the menopause (0.5 mg. = 1 R.U.)[2] and *c*) a crude preparation of menopause gonadotropin made in our laboratories. (5 mg. = 1 R.U.).

First, it was ascertained that these 3 preparations contained gonadotropin of hypophyseal origin by the weight curve of the ovaries and the gonadotropic reaction of Microtus guentheri, a muride which is refractory to small doses of prolan from pregnancy urine (3). Comparative investigations, as will be reported below, are practicable only if all of the gonadotropic substances used have been assayed and standardized in a uniform way. We chose the 3-week-old immature female rat for standardization of the preparations according to Zondek's original method (4, 5). One R.U. is the minimal amount of gonadotropic hormone which induces the anterior pituitary reaction known as APR I (vaginal estrus with full cornification). This unit is the most reliable and corresponds in our rat strain to 1/3 – 1/6 I.U.[3] According to this method we standardized the 3 preparations described above as well as 4 other preparations used for this assay: PU prolan[4] (1γ = 1 R.U.); placental prolan, 4 mg.

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[1] Aided in part by a grant of the Rockefeller Foundation.

[2] We are deeply indebted to E. R. Squibb & Sons, New York City, for the supply of Prospermine from male urine and Gamone from menopause urine.

[3] This has been recently confirmed by F. E. & M. C. D'Amour: *Endocrinology* 26: 93; 27: 68. 1940.

[4] Korotrin was generously supplied by the Winthrop Chemical Company, New York City.

= 1 R.U.; blood prolan, 3 mg. = 1 R.U.; pituitary gonadotropin 5 mg. = 1 R.U., prepared as described in 1937 (1). Twenty immature female rats were used for every standardization, that is a total of 140 rats for the 7 preparations.

The PU prolan antiserum had been obtained by the daily injection of 100 R.U. of PU prolan[4] into a female rabbit (twice weekly intravenously and 4 times subcutaneously). The antiserum was drawn 1 year after the beginning of the injections. It had been prepared as an acetone dry powder (6) and had an antigonadotropic titer of 0.1 mg. = 1 P.A.U. (6).[5]

Increasing amounts of urine prolan antiserum were mixed with 10 R.U. respectively of the following human gonadotropic preparations:

1. pregnancy urine prolan (korotrin)
2. male urine gonadotropin (prospermine)
3. menopause urine gonadotropin (gamone)
4. menopause urine gonadotropin (own preparation)
5. pregnancy blood prolan (own preparation)
6. placental tissue prolan (own preparation)
7. pituitary gland gonadotropin (own preparation)

Every assay was performed with 5 immature female rats according to our technic (1) as indicated in table 1. Thirty-five rats were used for the assay of the 7 preparations.

TABLE 1. ACTION OF 10 R.U. EACH OF VARIOUS GONADOTROPIC HORMONES WHEN MIXED AND INJECTED WITH PREGNANCY URINE PROLAN ANTISERUM INTO IMMATURE FEMALE RATS

PU Prolan Antiserum	Gonadotropic Reaction		
	APR[2] I Vaginal estrus	APR II Follicle hemorrhage	APR III Corpora lutea
PAU[1]	(1) PU prolan; (2) male urine gonadotropin; (3, 4)+menopause urine gonadotropin		
5	+	—	—
9	+	—	—
10	—	—	—
50	—	—	—
130	—	—	—
	(5) pregnancy blood prolan; (6) placental tissue prolan; (7) pituitary gland gonadotropin		
5	+	—	+
10	+	—	+
100	+	—	+
120	+	—	—
130	—	—	—

[1] Prolan-anti-unit; see footnote 5.
[2] APR = anterior pituitary reaction.

We checked our results by repeating the investigations in 5 series of assay each comprising 35 rats, the whole study comprising 175 rats. Since the results were the same in all 5 series the result obtained with the various types of preparations are presented in tabular form.

Table 1 demonstrates that 10 R.U. of male urine gonadotropin and 10 R.U. of menopause urine gonadotropin were inactivated, just as 10 R.U. of PU prolan had

[5] 1 PAU (1 prolan-anti-unit) is the smallest amount of antiprolan which inhibits the gonadotropic effect of 1 R.U. of prolan. Ten units at least should be assayed in one test rat and the minimal inhibiting titer be fixed in cc. of antigonadotropic serum or in mg. of the stable acetone dry powder (6).

been, by a small quantity (10 PAU) of an antiserum prepared against PU prolan. On the other hand, 10 R.U. of pregnancy blood prolan, placental tissue prolan and pituitary gland gonadotropin uniformly required a higher quantity of this antiserum for inactivation (130 PAU).

In the last series of assay we compared the gonadotropic reactions of our 7 gonadotropic preparations against the urine prolan antiserum using different doses. The vaginal estrus reactions (APR I) obtained in this series are summarized in table 2 and the 2 menopause urine gonadotropin preparations are noted in one column.

TABLE 2. GONADOTROPIC REACTION AFTER INJECTION OF A MIXTURE OF P.U. URINE PROLAN ANTISERUM WITH VARIOUS HUMAN GONADOTROPIC PREPARATIONS INTO IMMATURE FEMALE RATS

Pregnancy urine prolan antiserum	Gonadotropic reaction (APR I = vaginal estrus) of 10 R.U. of various gonadotropic hormones of human origin after mixture with antiprolan					
	pregnancy urine prolan	male urine gonadotropin	menopause urine gonadotropin	pregnancy blood prolan	placenta prolan	pituitary gonadotropin
PAU						
5	+	+	+	+	+	+
10	−	−	−	+	+	+
100	−	−	−	+	+	+
120	−	−	−	+	+	+
10	−	−	−	−	−	−

Table 2 demonstrates that the gonadotropin derived from the urine of normal males and from women in the menopause, were neutralized just as PU prolan had been by a PU prolan antiserum; whereas pregnancy blood prolan, placental prolan and hypophyseal gonadotropin, all non-urine derivatives, reacted differently. Ten R.U. of the urine preparations (even those containing pituitary gonadotropins) are inactivated by 10 P.A.U. of PU prolan antiserum—that is a 100% activity; on the other hand, 130 P.A.U. were required for the inactivation of gonadotropins from other human sources such as pregnancy blood, placenta and pituitary, which means a 7% antigonadotropin-activity only.

DISCUSSION

These results have a special bearing on the mechanism of antihormone formation. They show that the quantitative degree of antihormone specificity is not dependent upon the structure of the hormone itself, but only upon its source. As is well known, PU prolan is supposed to be of placental origin since it differs in many respects from male urine gonadotropin and menopause urine gonadotropin, which are supposed to be of hypophyseal origin. The present experiments show that the PU prolan antiserum is 100% effective against male urine gonadotropin and menopause urine gonadotropin. On the other hand, this serum has been shown to have an effectiveness of only 7% against human hypophyseal gonadotropin. This discrepancy together with the fact that the activity of the PU prolan antiserum towards pregnancy blood prolan and placental blood prolan (the supposed mother substances of PU prolan) is also only 7%, permits us to conclude that the decisive factor for antihormone specificity is not the origin (pituitary or placenta) nor the composition (urine prolan or hypophyseal gonadotropin) but only the source (urine or blood or organs) of the hormone used for immunization. As long as a gonadotropin is present in the pituitary body, blood or placenta it is only 7% inactivated by PU prolan antiserum; If, however, this gonadotropin passes through the kidneys into the urine it is 100% inactivated by the anti-

serum. This fact shows that the accompanying proteins of the gonadotropic hormones play a decisive part in antihormone specificity and this furnishes further support to the theory that antihormone formation is a process which is closely related to immunization phenomena.

SUMMARY

The antigonadotropic activity of a PU prolan antiserum has been tested against normal male urine gonadotropin and menopause urine gonadotropin preparations containing gonadotropic hormone of hypophyseal origin. The antigonadotropic activity in these cases was proved to be 100%. On the other hand, the antiserum revealed only a 7% activity against human placental prolan, human blood prolan and human pituitary gonadotropin. These results indicate that the specificity and the quantitative degree of the antiprolan activity does not depend upon the structure of the hormone itself (prolan, hypophyseal gonadotropin) but merely upon the origin (urine, blood, placenta or hypophysis).

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THE DESOXYCORTICOSTERONE ACETATE REQUIREMENT OF THE ADRENALECTOMIZED DOG

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THE ABILITY OF DESOXYCORTICOSTERONE ACETATE (1, 2) to maintain the life of adrenalectomized animals has been adequately demonstrated (3–5), and its usefulness in the treatment of Addison's disease has been described (6–8). A daily maintenance dose of 1 to 1.5 mg. in oil, injected subcutaneously, is said (4) to be sufficient for a 10 kg. dog on a lean meat, salt-free diet, as compared to 6 to 8 cc. of aqueous cortical extract. Kuizenga, Nelson and Cartland (9) have reported comparative assays on cortical hormone substances in the rat but, apart from the report of Thorn and Eisenberg (4) and a brief preliminary communication of our own (10), we are not aware of any papers describing such a comparison being made on the dog. We, therefore, have extended our earlier work and determined the minimal amount of desoxycorticosterone acetate[2] necessary to maintain adrenalectomized dogs on diets containing different amounts of NaCl and also have assayed two cortical extracts. In addition to this data, certain observations which indicate that the synthetic hormone does not effect complete replacement therapy will be reported.

TECHNIC

Seven adult male dogs, adrenalectomized in two stages, were used. They were kept in a heat-regulated room and allowed to run on the floor for 2 or 3 hours daily. The diet consisted of: lean, raw beef, 76 parts; cracker meal, 14 parts; bone meal with iron, 5 parts; butter, 3 parts; yeast, 2 parts. By analysis it contained approximately 1 gm.% NaCl. This was termed the "1% NaCl diet." In some experiments, NaCl was added to the food: 1 gm. per 100 gm. This was then termed the "2% NaCl diet." Adrenalectomized dogs showed signs of severe adrenal insufficiency 2 to 4 days following cessation of cortin injections when on the 1% NaCl diet, and in 7 to 10 days when on the 2% NaCl diet. Water, 100 cc., was added to each dog's ration to hold the ingredients together. The quantity of food given the dogs each day was sufficient to maintain body weight while they were on adequate therapy which was approximately 300 gm. for an 8 to 10 kg. dog. This was given at one feeding in the early afternoon and was always all eaten during the assay period. Consequently, food and salt intake constitute constant factors in the assay. Blood for estimation was drawn from the jugular vein in oiled syringes at weekly intervals, or oftener, at least 16 hours after food and the last hormone injection. Packed cell volume estimations were made on heparinized blood. The methods used in analysis

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[2] The desoxycorticosterone acetate used in this investigation was supplied by the Schering Corporation, Bloomfield, N. J., through the kindness of Dr. E. Schwenk. We are also indebted to Dr. David Klein of the Wilson Laboratory, Chicago, Illinois, for a supply of aqueous cortical extract.

of the blood have been described previously (11). The heart rate was obtained by auscultation with a stethescope while the animal was at rest.

The method of assay was basically that of Harrop, Pfiffner, Weinstein and Swingle (12) but, for reasons to be related later, their criteria had to be modified. These investigators defined a dog unit as the minimum daily kg. dose necessary to maintain body weight and prevent a rise in the non-protein nitrogen of 15 mg. or to a level above 50 mg. per 100 cc., when injections were given twice daily and reduced at 7 to 10-day intervals. Pfiffner, Swingle and Vars (13) modified this technic, taking a 100% rise in the blood urea and clinical symptoms as their criteria of insufficient dosage. They pointed out that marked insufficiency and death can occur at peak body weight. The 'dog method,' as either variant of the above technic has come to be called, has been used extensively by other research workers including Cartland

TABLE 1. ADRENAL CORTICAL HORMONE REQUIREMENT OF ADRENALECTOMIZED DOGS

Dog	Weight[1]	Aqueous Cortical Extract 2% NaCl Diet		Desoxycorticosterone Acetate		Remarks
		Conn. Lab.	Wilson	2% NaCl Diet	1% NaCl Diet	
		Daily minimal maintenance dose		Daily minimal maintenance dose		
No.	kg.	cc.	cc.	mg.	mg.	
1	10	0.5	...	0.469	...	Died suddely while on low dose.
2	11	2.0	0.5	0.469	...	Developed cardiac irregularities and died suddenly.
3	13	2.0	...	0.469	...	Died: hypoglycaemia after treated for crisis.
4	17	2.0[2]	0.5	0.234	1.87	
5	7	1.0	0.25	0.312	1.25	
6	8	1.0	...	0.30	1.87	
7	8	2.0[2]	...	0.30	1.25	

[1] Weight given to nearest kilogram; varied between assays.

[2] Dogs maintained adequately on this amount but assay not carried to end point for unavoidable reasons.

and Kuizenga (14) and Cleghorn and associates (15), who found 7 days a satisfactory period for each dosage level. For the present study it was felt that a longer period would result in greater accuracy in view of the slower absorption of desoxycorticosterone acetate from the vehicle in which it is dissolved (sesame oil). The injection period employed was 13 to 15 days, the dose being halved at the end of each period. All injections were given intramuscularly in the gluteal region. A 50% rise in non-protein nitrogen was provisionally chosen as the end point indicating insufficient dosage; 35 mg. % was selected as the normal level for purposes of this calculation. All dogs were restored to normal health and weight by adequate treatment after periods of insufficiency before starting a fresh assay.

RESULTS

The results of assays of both aqueous cortical extracts used and of desoxycorticosterone acetate are contained in table 1.

The average daily minimal maintenance dose of desoxycorticosterone acetate was 0.365 mg. per dog for the 7 dogs studied on the 2% NaCl diet. For the 5 dogs (No. 1, 2, 3, 5 and 6) the average dose was 0.404 mg. The assay of Connaught Laboratories' cortical extract obtained on these dogs showed an average requirement of 1.3 cc. per dog per day. On this basis 1 cc. of the commercially available desoxycorticosterone

acetate (5 mg.) is equal to 16 cc. of this aqueous extract. The average daily requirement of the Wilson product was 0.42 cc. for the 3 dogs in this study, as compared to 0.305 mg. of the synthetic hormone estimated on the same animals. Assayed in this way, 5 mg. (1 cc.) of desoxycorticosterone acetate is the equivalent of 6.8 cc. of the Wilson extract. Comparison of the desoxycorticosterone acetate requirement on the 2 and 1% NaCl diets, respectively, can be made from the results on *dogs* 4, 5, 6 and 7, in which both assays were done. An average requirement of 0.286 mg. per day was obtained on the 2% NaCl diet; 1.56 mg. per dog per day on the 1% NaCl diet.

Estimation of Hormone Requirement

Aqueous Cortical Extract Assay. In all dogs a sharp rise in the NPN occurred on an inadequate dose. The NPN rise was more than 90% by the time the assay was

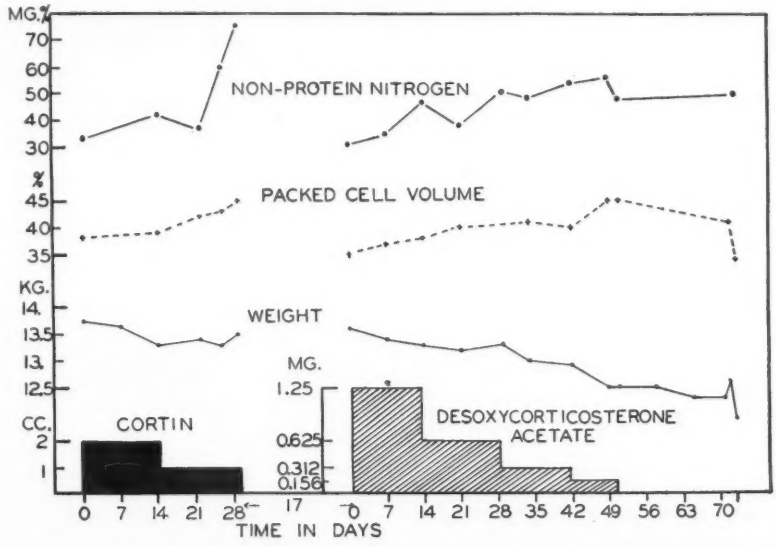

Fig. 1. Findings in *dog* 3. April 30, 1938, left adrenal removed. Jan. 23, 1939: cortin assay begun 2% NaCl diet; Feb. 21, 1939, cortin assay ended; Feb. 28, 1939; desoxycorticosterone acetate assay begun, 2% NaCl diet; April 28, 1939 (42nd day), blood Na 148 m. eq.; K 5.6 m. eq.; May 4, 1939 (50th day), desoxycorticosterone acetate injections stopped; dog seemed normal though weight at lowest point so far and packed cell volume increased; May 8, 1939 (54th day), dog seemed slightly weak; May 12, 1939 (58th day), heart rate 74 on one occasion, 132 on another; on succeeding days, resting heart rate varied from 64 to 80 or from 120 to 130; May 23, 1939 (69th day), some food vomited; May 24, 1939 (70th day), seemed weak; May 25, 1939 (71st day), found collapsed on floor at 11.00 A.M., heart rate 60; electrocardiogram taken; treatment given. Animal died next day in hypoglycaemia. Minimal maintenance dose of desoxycorticosterone less than 0.625 mg. daily and more than 0.312 mg., about 0.469 mg.

terminated in all but one case, taking 35 mg. % as normal. In the one exception it was only 60%. When the increase in NPN was first observed, a rise in the volume of packed red blood cells of about 13% (5 points) was also usually found (fig. 1). With one exception (*dog* 1) a loss of at least 400 gm. from the peak weight was evident by this time. These changes generally occurred within the first week of reduction of the dose from the minimal maintenance level. When these signs of insufficient dosage were first noted, the same amount of hormone was continued for a few days after reduction of the dose and the observations were then repeated. Deviation from

the normal findings was confirmed in every case. In no instance did any serious clinical sign of adrenal insufficiency, such as anorexia, vomiting, bradycardia, weakness or collapse, appear prior to the NPN, haematocrit or weight changes. Only *dog 4* showed any other sign of adrenal insufficiency (bradycardia) during the period of inadequate dosage following the significant rise in NPN. Recently 3 adrenalectomized dogs have been maintained for weeks on a sub-optimal dose of cortical extract (16). Despite elevated NPN values of 60 to 100 mg. %, a marked cell volume increase and weight loss, no serious clinical sign of insufficiency developed. The minimum maintenance dose in dogs receiving aqueous extract was judged to be that amount which

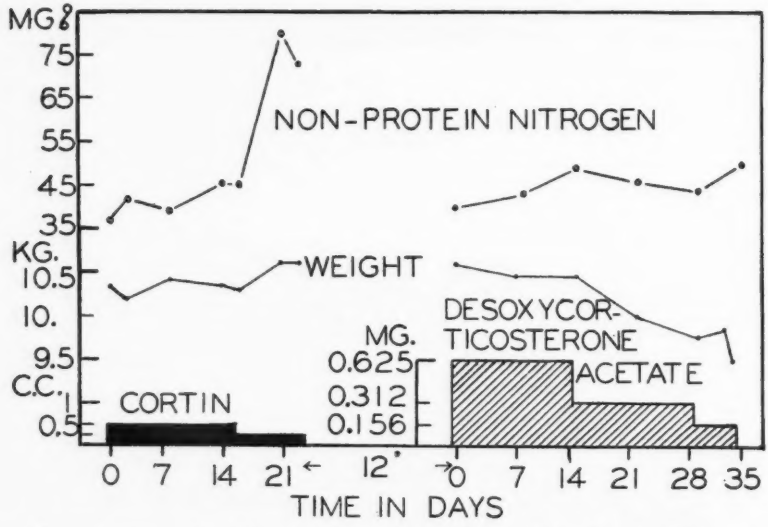

Fig. 2. Findings in *dog 1*. May 30, 1938, second adrenal removed. Jan. 8, 1939, cortin assay begun; March 16, 1939, cortin assay terminated; March 21, 1939, desoxycorticosterone assay begun, 2% NaCl diet; April 25, 1939 (29th day), dog eating and behaving normally, though weight loss marked, blood values normal except for serum Na of 139 m. eq.; May 1, 1939 (34th day), animal bright and frisky, though weight at lowest point so far; May 2, 1939 (35th day), found dead but still warm, undigested vomitus in cage, blood from heart: NPN only obtained, 50 mg. %. *Post mortem examination.* Lungs and heart normal in the gross; stomach, marked congestion of the mucosa, 2 ulcers near pylorus; duodenum, slight congestion; adrenal sites clear.

an animal was receiving prior to the reduction of the dose to the level on which the significant blood changes occurred.

Desoxycorticosterone Acetate Assay. In dogs receiving the 2% NaCl diet the NPN did not provide so sharp an end point as in the cortical extract assay. It rose slowly as the dose was reduced but only in 5 of the 7 dogs studied did the rise exceed 50% of the normal. An increase in packed cell volume and weight loss occurred as the dose was reduced. These changes became even more marked with further reduction in the amount of hormone given though the NPN did not rise further in the majority of cases. Figure 1 illustrates these points.

Weight loss and NPN must be considered of equal importance in assessing the minimal maintenance dose of desoxycorticosterone acetate. This is suggested by the above findings and is supported by the fact that *dog 1* (fig. 2) died when the only definite sign of insufficient dosage was a marked weight loss. In most cases increase in packed cell volume occurred simultaneously. Consequently we elected to utilize

weight loss and change in packed cell volume, in addition to a 50% increase in NPN, as criteria of the adequacy of desoxycorticosterone acetate therapy. A 400 gm. weight loss and 5 point increase in haematocrit were judged to be significant changes. When significant changes occurred in two of the three criteria, the dose was considered to be insufficient. In 4 of the assays (*dogs 1, 2, 3, 4*) two of the three criteria just fell within the lower limit of this arbitrary standard of inadequacy on a certain dose. The minimal maintenance dose was then designated as the average between that on which these changes occurred and the previous dose on which only one or no change occurred. In the other assays the inadequate dose was indicated by practically concurrent and definitely positive changes in all three criteria. The immediately preceding dosage level, on which only one or no change was observed was considered the minimal maintenance dose in these cases.

Some difficulty was encountered in deciding the packed cell volume and weight levels from which calculation for significant change should be made. Large doses of desoxycorticosterone lead to considerable hydration of the animal and to an abnormally low cell volume and increased weight. Calculations based on such artificially low and high figures, respectively, would only yield fictitious results. Therefore it was found necessary to take an average of several consecutive values obtained at weekly intervals when the dog was being maintained in a relatively stable state.

Whether the minimal maintenance dose arrived at on the basis of the 2-week assay period represents the maintenance dose for longer periods is answered roughly by further results obtained on *dogs* 6 and 7. These animals each had a pellet of desoxycorticosterone acetate (approximately 100 mg.) implanted subcutaneously by Thorn's (5) technic for periods of 45 and 67 days respectively. Both animals received the 2% NaCl diet and were maintained adequately on this form of therapy, as evidenced by all criteria. Both animals went into severe adrenal insufficiency when the pellet was removed. The absorption rate of the pellets was found to be 0.39 mg. a day in both dogs. This is but 30% more than the minimal maintenance dose by injection. It is possible that smaller pellets might have effectively maintained the animals.

The assay of desoxycorticosterone acetate gave more clear cut results when the 1% NaCl diet was used. With reduction in the amount of hormone administered the packed cell volume rose sharply and a significant amount of weight was lost. This was followed in a few days by a steep rise in the NPN, which occurred without further reduction in the amount of hormone administered in 2 cases and after reduction of the dose in 2 others. This rise exceeded 85% in all 4 cases by the time the assay was terminated. The criteria for estimating the minimal maintenance dose of desoxycorticosterone acetate were the same as applied on the 2% NaCl diet. For *dogs* 4 and 6 the maintenance dose was estimated as being the average of the dose on which two of the criteria were positive and the preceding dose on which no change occurred.

Death of Dogs Receiving Desoxycorticosterone Acetate

It will be noted in table 1 that 3 of the dogs used in the investigation died. They were receiving desoxycorticosterone acetate at the time death occurred. In no case was infection an apparent factor. In all three instances the circumstances attending death were unusual. The history of *dog 1* is contained in the legend of figure 2; it died suddenly while on a low dose of desoxycorticosterone acetate. The only definite indication prior to death that the replacement therapy was inadequate was a decline in weight. Admittedly, the blood Na (139 m. eq.) was a little low on April 25, but the potassium value (5.6 m. eq.) was normal. The post mortem findings were char-

acteristic of adrenal insufficiency, though less marked than in some cases. This animal had a very low cortin requirement (table 1). It is impossible to say what function failed and led to death. In view of subsequent findings in other animals, it seems probable that the heart may have been at fault.

The second instance of death of an animal while on desoxycorticosterone acetate is detailed in the following protocol:

Dog 2: Sept. 10, 1938, second adrenal removed. Jan. 8, 1939, cortin assay begun. Feb. 28, 1939, desoxycorticosterone acetate assay begun on 2% NaCl diet. Severe adrenal insufficiency on the 7th day after last injection. Heart rate 72; restored by intravenous hypertonic saline and glucose to 130. May 17, 1939, cortin assay. July 18, 1939, cortin maintenance. Aug. 25, 1939, desoxycorticosterone assay on 1% NaCl diet started: 2.5 mg. daily. Sept. 3, 1939, dog lethargic; some food left; no weight loss; heart rate 60; blood serum Na 148 m. eq.; K 5.5 m. eq.; NPN 38 mg. %; packed cell volume 44%. Hypertonic saline and glucose given intravenously. Heart rate returned to normal range, temporarily only. Sept. 4, 1939, desoxycorticosterone 5 mg. daily. Sept. 7, 1939, desoxycorticosterone cut to 3.75 mg. daily; 1% NaCl diet. Sept. 13, 1939, dog eating well; weight normal; blood serum Na 153 m. eq.; potassium 6 m. eq.; NPN 33 mg. %; blood sugar 80 mg. %; packed cell volume 35%. Heart rate varies between 72 and 80. Electrocardiogram shows an irregular rhythm with many *P* and *T* waves absent. Sept. 15, 1939, cortin (Connaught Laboratories), 1 cc. twice daily; 2% NaCl diet started. Sept. 20, 1939, heart rate still low but dog seems fairly well. Cortin (Connaught Laboratories) 4 cc., twice daily, and 1% NaCl diet begun. Sept. 26, 1939, heart rate now in normal range: about 130. Sept. 27, 1939, heart rate 144. No cortin; four pellets of desoxycorticosterone acetate (about 120 mg. each) implanted subcutaneously. Dog ate some food. Sept. 28, 1939, animal found dead.

Post mortem findings: Stomach—extensive congestion and ulceration of mucosa; mucosa of duodenum and large bowel congested. Pancreas beefy red. Heart soft; area at tip of left ventricle suggestive of infarction. Sections in this area showed degeneration and necrosis of cardiac muscle, edema and constriction of smaller coronary vessels though no definite thrombosis found.

The deterioration in the condition of this animal on September 3, 1939, was marked by the association of lethargy, anorexia, an increased packed cell volume and a bradycardia.[3] The NPN and blood electrolytes were within normal limits. It seems reasonable, therefore, to suggest that cardiac arrhythmia was the cause of the other signs of adrenal insufficiency. Though bradycardia has been reported by numerous workers as a characteristic finding in dogs in severe adrenal insufficiency, and in our experience it is present in at least 80% of such animals on the 2% NaCl diet, we are not aware that it has been observed previously in dogs showing normal electrolyte values. The blood findings indicate that insofar as the electrolyte metabolism was concerned the treatment was adequate. In fact, the dog was receiving 30% more desoxycorticosterone acetate than the largest minimal dose required for the maintenance of other animals on the 1% NaCl diet. Despite this the cardiac arrhythmia developed so that, in this respect, the treatment was inadequate.

Intravenous injection of hypertonic saline and glucose restores the cardiac rhythm to normal in dogs in adrenal insufficiency. It did so in the case under discussion on Sept. 3, 1939. The effect of the intravenous injection on the heart was not lasting, though it brought about a lowering of the packed cell volume. The blood chemical values persisted in the normal range. Although the heart rate did not fall to 60 again, counts of 72 to 80 were observed frequently, and an electrocardiogram taken on

[3] The normal resting heart rate in most of our adrenalectomized dogs adequately maintained on the 2% NaCl diet is frequently in the 110 to 130 range. This is higher than reported by other workers and will be discussed in detail elsewhere.

Sept. 13 showed an irregular rhythm and absent *P* waves. Despite this the dog seemed fairly well though not so energetic as usual. Restoration of the heart rate to the range of 130 to 145 beats per minute, normal for this dog, occurred only following injections of Connaught Laboratories' cortin (8 cc. in bi-daily doses) for 6 days, while continuing the 1% NaCl diet. Death occurred 40 hours after the cessation of cortin injections and 36 hours after the implantation of pellets under local anesthesia. The pellets, if absorbed at the rate obtaining in other animals investigated in this laboratory, would have liberated 1.5 mg. desoxycorticosterone acetate daily.

In view of the cardiac irregularities observed in this dog, it seems likely that some change in the function of the heart led to its death. The pathological appearance of the heart, both in the gross and microscopically, indicates that this change was accompanied ultimately by an organic change of the nature reported by Hall and Cleghorn (17).

The preliminary history of the third animal failing to respond adequately to desoxycorticosterone treatment is described in the legend of figure 1. The following details are particularly pertinent to the present discussion.

> Following cessation of desoxycorticosterone acetate injections, this dog showed periodic variations in heart rate varying from 64 to 80 and 120 to 130. Vomiting occurred on the 19th day after the last injection, weakness on the 20th, and collapse on the 21st day. An electrocardiogram taken on the 71st day, with the heart rate at 60 beats per minute, showed absent *P* waves and frequent coupled beats. The NPN was only 50 mg. %, the blood sugar 100 mg. %, and the packed cell volume 41% which was less than that at the time injections were stopped. Intravenous hypertonic saline and glucose and 7 cc. cortical extract (Wilson) restored the cardiac rate to 120 within 2 minutes. Desoxycorticosterone acetate, 5 mg., was given intramuscularly, and a similar dose again in the evening. At 3 o'clock in the afternoon the electrocardiogram was normal. The dog ate 200 gm. of food. May 26, 1939, (72nd day) the animal seemed weak in the morning so intravenous-hypertonic saline and glucose were given with 5 mg. desoxycorticosterone acetate intramuscularly. At 5.00 P.M. the dog was found prostrate, but with heart still beating. Examination of blood from the heart showed: packed cell volume 34% and blood sugar 20 mg. %. *Post mortem examination:* heart, no evidence of infarction; stomach mucosa congested and one small ulcer present; duodenum, slight congestion only; ileum not congested; petechial haemorrhages in mucosa of large bowel; kidneys engorged; pancreas intensely congested; adrenal sites clear.

The bradycardia observed in this animal occurred periodically in the 21 days which elapsed between the cessation of injections of desoxycorticosterone acetate and the final collapse. The slow rate returned spontaneously to normal except on the last occasion, on May 25, when it was associated with collapse. Then an intravenous injection of hypertonic saline and glucose was followed by a speeding up of the heart rate to 120 per minute and, though the electrocardiogram showed this was due to the induction of ventricular tachycardia, a normal rhythm must soon have ensued. Three hours later, when another electrocardiogram was taken, the rhythm was normal. Electrolyte studies were not made on this occasion but the NPN was not very high and the blood sugar was within normal limits; the packed cell volume had actually declined from the high levels obtaining 3 weeks earlier. It seems, therefore, that the collapse was due to the cardiac change. The death of the animal in hypoglycaemia, on the next day, was probably related to the intravenous glucose injection given that morning. We have many times given saline and glucose to animals in adrenal insufficiency and have never seen hypoglycaemia develop thereafter. However, in such animals plenty of aqueous extract was always given. In this case, aqueous extract was not given after the emergency treatment the day before the

animal died nor did the dog eat on the day of death. It should be noted, however, that on May 25, the day of the initial collapse, an amount of desoxycorticosterone equivalent to approximately 26 times the daily minimal maintenance dose (on the 2% NaCl diet) was given in addition to about 14 times a maintenance dose of cortical extract. On the following day when death occurred, 13 times the maintenance dose of desoxycorticosterone was given in the morning at the time of the intravenous injection of saline and glucose. Our routine treatment of animals in adrenal insufficiency consists of the administration of but 7 to 15 times the maintenance dose of cortin on the day of collapse along with the intravenous therapy, and of 5 to 10 times the maintenance dose the following day. In dogs so treated, we have never encountered the development of hypoglycaemia.

The 21 days which elapsed between the cessation of injections and desoxycorticosterone and collapse in this animal was about three times longer than that usually taken for the development of critically severe adrenal insufficiency after cessation of hormone therapy on the 2% NaCl diet. It is suggested that this was due to pocketing of the injected oily solution of desoxycorticosterone which resulted in a longer period of absorption that continued after cessation of injections.

DISCUSSION

Aqueous cortical extracts assayed over a 2-week period for each dosage level give a potency value which is probably less than that which would have been obtained with the usual assay period of 7 days. The actual minimal maintenance dose, however, is more closely approximated by using the longer period. Our results indicate that the Wilson extract is a very potent preparation. This product has been improved since our experience with it in early 1939 (18). The Connaught Laboratories' extract also has been considerably fortified recently, so that the findings reported here do not represent the strength of the product on the market now which is 2 to 3 times more potent. The minimal daily maintenance dose of desoxycorticosterone acetate found for the 1% NaCl diet agrees roughly with that of Thorn and Eisenberg (4) using a diet of similar electrolyte content.

The cortical extract requirement of the dogs was found to vary to much the same extent as found by Pfiffner, Swingle and Vars (13). Only in the case of *dog 1* was the maintenance dose markedly less than that of the others. The desoxycorticosterone requirement of this animal was not correspondingly low and it died suddenly while receiving a small daily dose. Conversely, *dog 4* had a markedly low desoxycorticosterone requirement which was not paralleled by a similarly low cortical extract requirement, at least on the Wilson extract which was the only one effectively tested.

These findings indicate that there is no close parallelism in the variations of cortical extract and desoxycorticosterone requirement in the dogs studied, nor is the requirement closely related to the size of the animal. Furthermore, one cannot draw any conclusion as to the amount of desoxycorticosterone or desoxycorticosterone-like substances in the cortical extracts on the basis of these observations. The faster absorption and more evanescent action of the aqueous extract renders it less efficient and, for that reason, it would appear that a greater hormone content would be necessary to produce a comparable effect. Evidence that this is not necessarily so has been presented by Mason (19) who has found that the amorphous residue of cortical extract contains material many times more potent than desoxycorticosterone acetate.

The more clear-cut end point in the aqueous extract assay, insofar as the NPN rise is concerned, probably is due to the transient presence of the hormone. The slower and less marked rise of the NPN in the desoxycorticosterone-treated animals on the 2% NaCl diet and the occurrence of crises with the NPN but little elevated resemble the conditions in Addison's disease cases in which a high NPN is not usual

in crisis. Nitrogen retention is not apparent so early in animals receiving inadequate doses of desoxycorticosterone as is weight loss. Under the conditions of the experiment the latter could only have been due to salt and water loss.

A more definite assay of desoxycorticosterone acetate is obtained using the diet of lower salt content (1% NaCl). Presumably this is so since the physiological disturbances develop more rapidly when less salt is available.

Although the packed cell volume usually increased markedly on an inadequate dose of desoxycorticosterone or of cortical extract, it is apparent in *dog* 3 (fig. 2) that the packed cell volume value had actually decreased at the time collapse occurred. We have observed this phenomenon in other animals receiving an inadequate dose of hormone over a long period and can only explain it as being due to stasis of cells or to the development of anemia. It would appear, then, that some factor other than hemoconcentration must have been predominant in the crisis in *dog* 3. Since the blood sugar was within normal limits, it seems probable that cardiac arrhythmia was the responsible factor. The heart rate was 60 at this time. *Dog* 2 had the same heart rate while in crisis on September 3, 1939. In the latter animal a rate of 72 was present at the time of the crisis on February 28, 1939. In both animals, and in others we have observed, counts of 70 to 80 were made when the animals were eating and behaving normally, normal rate for these animals being 115 to 130. There seems, therefore, to be some significant functional difference in the cardiac efficiency at different times with these low rates. There is not yet sufficient electrocardiographic evidence to say what that is. It is not complete heart block which occurs in some animals in advanced adrenal insufficiency (17, 20).

It would seem from the evidence obtained on *dog* 2 that disturbance in blood electrolytes is not necessary to the development of cardiac arrhythmias in adrenalectomized dogs. Nevertheless, the intravenous injection of hypertonic solutions restored the rhythm to normal. This did not last long and it was only after the daily administration of 8 cc. of cortin (Connaught Laboratories) for 5 days that the rate rose from the 70 range to 130. On the assay basis, this amount of cortin is equal to only 2 mg. desoxycorticosterone daily. The dog had been receiving 2.5 mg. daily when the arrhythmia developed. It seems, therefore, that aqueous cortical extract contains a factor, or factors, which influences cardiac function favourably, and which apparently is not present in desoxycorticosterone acetate. Of especial interest in this connection is the report of Zwemer and his associates (21, 22) that cardiac glucosides favour survival of adrenalectomized cats and protect against potassium poisoning.

The death of *dog* 3 occurred some 6 or 7 hours after an intravenous injection of saline and glucose. The treatment given the previous day had restored the animal so that it ate 200 gm. of food in the afternoon. The intravenous injection on the morning of the day of death insured that any electrolyte disturbance had been adequately remedied. The amount of desoxycorticosterone administered insured the retention of salt and water and diluted the blood, as evidenced by the packed cell volume of 34%. Yet it did not prevent the development of fatal hypoglycaemia. Thorn and his colleagues (23) have shown that desoxycorticosterone acetate therapy does not restore the abnormality in carbohydrate metabolism present in adrenalectomized animals or in patients with Addison's disease. Fasting or intravenous glucose therapy may be followed by hypoglycaemia in desoxycorticosterone-treated subjects; cortical extracts prevent this.

Our results indicate that, under optimal circumstances, adrenalectomized dogs usually can be effectively maintained by daily injections or subcutaneously implanted pellets of desoxycorticosterone acetate. We have restored animals showing a mild degree of insufficiency without cardiac irregularities by injections of desoxycorticosterone alone (unpublished observations).

SUMMARY

The amount of desoxycorticosterone acetate necessary to maintain an adrenalectomized dog has been determined in seven animals. On a diet containing approximately 2% NaCl the daily average minimal maintenance dose was found to be 0.365 mg. Compared to the aqueous cortical extracts also assayed, 5 mg. (1 cc.) of desoxycorticosterone acetate is equivalent to 16 cc. of one extract and 7 cc. of the other. The requirement on a diet containing a low amount of salt (1%) averaged 1.56 mg. or 5.5 times as much as the minimal maintenance dose of these dogs on the 2% NaCl diet. Three dogs died while receiving desoxycorticosterone acetate, one in hypoglycaemia following adrenal insufficiency otherwise adequately treated, one after showing persistent cardiac irregularities, and the other suddenly when apparently well except for some loss of weight. The interpretation of these findings is discussed and it is concluded that desoxycorticosterone does not effect complete replacement therapy, lacking some factor present in aqueous extracts necessary for carbohydrate and cardiac metabolism.

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A STUDY OF THE COLORIMETRIC DETERMINATION OF URINARY ANDROGENS[1] BY A MODIFICATION OF THE OESTING-NEUSTADT METHOD

Effect of Change of Temperature, Concentration and Solvent

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THE METHOD OF NEUSTADT (1) for the colorimetric determination of urinary androgens is virtually that of Oesting (2), except that the final estimation is made with a photoelectric colorimeter instead of a special visual instrument. In Neustadt's paper there is no mention of the necessity for temperature control. The method of Oesting which is used for color development has been discussed by Friedgood and Whidden (3). We had originally chosen the Neustadt method because of its apparent simplicity for clinical use, and had determined androgens routinely on a number of cases. In view of the anomalies observed by McCullagh, Schneider, and Emery (4) and by Talbot, Butler, MacLachlan, and Jones (5) in working with urine extracts and using more elaborate and sensitive procedures, it seemed worthwhile to us to attempt to alter the Neustadt-Oesting technic, standardizing the conditions yet retaining the essential simplicity of the operations. We studied first the effect of temperature on the determination of pure androsterone[2] and of various urinary extracts. We also investigated the effect of increasing the alcohol concentration of the solvent from 60 to 95%. In ascertaining the effect of temperature and change of solvent we also hoped to obtain differential values which might give some indication as to the nature of the reacting steroids in the urine extracts from various types of cases.

In this work, deviations from Beer's law were frequently noted in working with the urine extracts. The recent report of Friedgood and Whidden (6) regarding the influence of water on the Zimmermann reaction, and that of Friedgood and Berman (7) concerning deviations from Beer's law which they noted with urine extracts, has led us to amplify the part of this paper discussing such effects.

Apparatus. A Fisher photo-electric colorimeter,[3] was employed. This is a single photocell instrument employing a null method. The reading in percentage absorption is made from a dial. This reading is subtracted from 100 to give percentage transmission. The instrument is not adaptable to test tubes, so that the contents of the reaction tube must be transferred to a rectangular 10 mm. cell (capacity about 20 cc.) for

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[1] The term androgens is used for want of a more suitable descriptive term, for those urinary neutral fraction components giving the Zimmermann reaction.

[2] We are indebted to Dr. Erwin Schwenk of the Schering Corporation, Bloomfield, N. J., for the crystalline androsterone used in this work.

[3] Catalog No. 7-089, battery operated.

reading. The time consumed in transferring to the absorption cell, setting the instrument against solvent in a matched cell, and finally balancing to null point on the galvanometer is not a serious handicap for this reaction, for the sample may be taken from the water bath about 2 minutes before time to read without appreciable error. The dial of the potentiometer circuit is marked off in 0.5 divisions, and may be read to 0.2 divisions. We have periodically checked the instrument against glass filters as standards and have noted no variation in over a year. Range 1 of the instrument was employed in all determinations.

A filter,[4] 4 mm. thick, was employed in most of this work. The transmission of this filter is given as maximum at about 520 mμ.

The thermostated water-bath employed held the temperature within 0.2° C.

Procedures. The urine extracts used in this work were prepared by the usual 15-minute acid hydrolysis, using 10% HCl, followed by extraction with butyl ether (8, 9) in a separatory funnel. The combined butyl ether extracts were then treated with 10% NaOH, washed with water, and evaporated under reduced pressure. The residues were taken up with ether which was evaporated, and the material stored dry until determination. No charcoal was employed because of the losses reported in such practice by Talbot and Langstroth (10).

Separations, where made, into ketonic and non-ketonic fractions were carried out by the technic of Talbot, Butler and MacLachlan (11).

On occasion we wished to determine androgens and sodium pregnandiol glucuronidate by the method of Venning (12) in the same urine. The urine was extracted with butyl alcohol and the residual urine was acidified and treated as usual for androgens. Considerable chromogenic material was found in this previously extracted urine on colorimetric assay. The NaOH and wash water, and the first and second acetone fractions after the removal of the sodium pregnandiol glucuronidate by the usual precipitation technic of the Venning method were saved. The acetone fractions were evaporated down, water was added and the material re-heated to drive off residual acetone. The residue from the acetone fractions after this treatment and the NaOH and washings were all taken up in 500 cc. of water. Ten % by volume of concentrated HCl was added and the material was then treated as indicated above.

For the colorimetric procedure the dry residues obtained were taken up in 100 cc. of 95% alcohol and if necessary an aliquot was further diluted. When it was desired to use the same extract for several runs (*case B.R.*) it was stored from one time to the next by evaporating the alcohol solution to dryness again in an Erlenmeyer flask.

To Pyrex test tubes 250×25 mm. various amounts of the stock solution were added with a pipette. Additional alcohol was added for the 95% alcohol runs to make 20 cc. total volume; in the 60% alcohol runs water and 95% alcohol were added to make 20 cc. total volume of 60% alcohol. The tubes were placed in the thermostat for 20 minutes, then reagents were added, the development periods being spaced to allow time for the manipulations in reading the colorimeter. To the *A series*, for the measurement of the pigment intrinsically in the specimen (as affected by KOH and alcohol), were added 4 cc. of 95% alcohol and 4 cc. of 15% aqueous KOH. To the *B series*, for the estimation of the chromogen, 4 cc. of 2% m-dinitrobenzene in 95% alcohol solution and 4 cc. of 15% aqueous KOH were added. Blanks were always run for both series, those for the *A series* being negligible. The color development period was in all cases 1½ hours. There was practically no exposure to light during

[4] Corning #401 lantern-green filter. The instrument is sufficiently sensitive to permit the use, if care is taken, of the Rubicon #520 filter. It is, however, not sensitive enough to permit use of the Corning #511 (or Rubicon #420) filter, so that readings for correction purposes [cf. Talbot et al. (5)] could not be made.

this time. For routine work the year around we found 25° C. to be impractical as a temperature because of the location of the room; 30° C., however, was satisfactory.

For routine determinations the extract of 500 cc. of urine was taken up in 60% alcohol; of this 2.5 and 5.0 cc. portions were used. The reported value is based on the mean of the 2 results at different concentrations. The basis for the choice of these low concentrations was our finding of deviations from Beer's law discussed later in this paper.

Reagents. For the early work, Eastman T99 m-dinitrobenzene was purified essentially according to the method given by Neustadt (1) save that clarification with charcoal was also employed. Later, Eastman 99 grade was used. However, as a precaution for most of the data reported here we purified the Eastman 99 grade by the method of Callow, Callow and Emmens (13) and made up the 2% solution fresh each time. With the 60% alcohol method in our experience the purity of the m-dinitrobenzene is not a critical factor; also we have a set of data showing agreement in determining a urine extract when all of the grades mentioned above were used. KOH was made up fresh each time for most of the work, 15 gm. of the pellets (Mallinckrodt A. R.) to 100 cc. of water. Ninety-five % alcohol was of the usual USPX 190 proof grade. Butyl ether (Union Carbide and Carbon) was always first distilled under reduced pressure after treatment with alkali. Butyl alcohol (Mallinckrodt A. R.) was used without purification. Ether for anesthesia was used.

Crystalline androsterone[2] was used without further purification. It was dissolved in 95% alcohol.

RESULTS

The results with pure androsterone are given in table 1. In calculating K, the blank value was subtracted from the quantity $2 - \log (100 - D)$, then the result was divided by the number of mg. of androsterone per cc. of the final colored solution which is the number of mg. indicated in the table divided by 28. For each condition of temperature and concentration of alcohol the data obtained at different times are grouped separately and the order of these groups for the conditions specified is chronological. These data were obtained over a period of several months and concurrently with the studies of urine extracts. The alcohol concentrations indicated are those in the mixtures before addition of reagents; the effective concentrations during the reaction period are less; approximately 57 and 82% corresponding to the listed 60 and 95%.

As noted by other investigators the intensity of the color increases with temperature increases and with the concentration of alcohol employed. The intensity of the blank likewise increases markedly. From averaging the data listed, we find K to be 2.70 at 30° C. with 60% alcohol, 3.82 at 30° C. with 95% alcohol, 1.93 at 25° C. with 60% alcohol, and 2.82 at 25° C. with 95% alcohol.

From the data of table 1, it appears that androsterone obeys Beer's law under the conditions used. Examination of the data of Neustadt (1) at 'room temperature' also shows such obedience, likewise the data of Saier, Warga, and Grauer (14). The data of these investigators cannot be compared quantitatively with ours because of the different filter (Rubicon #520) and the test-tube absorption cell principle of the Evelyn colorimeter, which was used by both. Saier et al. also have data on dehydroisoandrosterone. Their K value for this substance is 1.6 times that for androsterone; their values for K of the 2 substances by the Friedgood-Whidden method (15) have a ratio of 1.3; by the method of Talbot et al. (11), K is the same. These ratios parallel the water content of the reaction mixture. Saier and Grauer (16) have values for mixtures of androsterone and dehydroisoandrosterone determined by the Neustadt method; these likewise obey Beer's law.

In tables 2 and 2A are listed the results obtained in studying urinary extracts under the same conditions as used for pure androsterone. Brief information concerning each case (the sex, age and the condition if differing greatly from the normal) is given in the heading of each tabulation. The cases whose condition is not indicated are, however, not necessarily to be considered normals; most were hospitalized and suf-

TABLE 1. RESULTS WITH PURE ANDROSTERONE; Corning #401 Filter, 10 mm. Cell

Androsterone	D	2-log (100-D)	K	Androsterone	D	2-log (100-D)	K
mg.				mg.			
Room temperature, 60% alcohol				30° C. 60% alcohol			
0.00	2.2	0.0097	—	0.00	4.0	0.0177	—
0.20	6.7	0.0301	2.86	0.40	12.4	0.0575	2.79
0.50	12.7	0.0590	2.76	(30.3°) 0.60	16.5	0.0783	2.83
0.90	21.0	0.1024	2.88	1.00	24.0	0.1192	2.84
1.90	36.7	0.1986	2.78				
3.60	57.0	0.3665	2.78	0.00	4.0	0.0177	—
4.50	64.9	0.4547	2.77	0.36	11.2	0.0516	2.64
7.00	81.9	0.7423	2.93	0.54	15.0	0.0706	2.74
25° C. 60% alcohol				0.00	3.8	0.0167	—
				0.50	13.4	0.0625	2.57
0.00	3.0	0.0132	—	0.50	13.7	0.0640	2.65
0.40	9.2	0.0419	2.01	1.00	22.9	0.1129	2.69
0.60	11.6	0.0535	1.88				
1.00	16.5	0.0783	1.82	0.00	2.9	0.0128	—
				0.41	11.2	0.0516	2.65
0.00	2.9	0.0126	—	0.41	11.3	0.0521	2.68
0.50	10.2	0.0467	1.91				
1.00	17.6	0.0841	2.00	0.00	3.2	0.0141	—
				0.71	16.8	0.0799	2.60
0.00	2.4	0.0106	—	1.41	28.9	0.1481	2.66
0.71	13.0	0.0605	1.97				
1.41	22.2	0.1090	1.95				
27.8° C. 60% alcohol							
0.00	3.4	0.0150	—	Blank values are usually means			
0.40	11.1	0.0511	2.53				
0.60	14.0	0.0655	2.36				
1.00	20.5	0.0996	2.37				
25° C. 95% alcohol				30° C. 95% alcohol			
0.00	7.8	0.0353	—	0.00	11.5	0.0531	—
0.50	18.1	0.0867	2.88	0.50	24.4	0.1215	3.83
1.00	27.0	0.1367	2.84	1.00	35.6	0.1911	3.86
0.00	6.4	0.0287	—	0.00	9.1	0.0414	—
0.56	17.9	0.0857	2.84	0.56	24.0	0.1192	3.90
0.85	23.0	0.1135	2.79	0.85	30.1	0.1555	3.76
1.13	28.1	0.1433	2.84	1.13	36.3	0.1959	3.83
2.12	41.8	0.2351	2.72	2.12	52.6	0.3242	3.74

fering from non-endocrine complaints of various types which might or might not affect the usual excretion of the compounds to be determined. In the tabulations in table 2 the first column gives the temperature, the second the concentration of alcohol before addition of reagents, the third indicates the amount of the total solution taken, the fourth the treatment as described previously under 'Methods,' the fifth the dial reading (D), the sixth the quantity 2-log (100-D) derived from D, the seventh (L_c) the quantities of the sixth column minus the reagent blank, the eighth (L_{1c}) these

quantities further corrected by subtracting the 'intrinsic pigment' values, and the last gives the value which would be reported using the values of K for pure androsterone previously given (table 1). To conserve space all these quantities are given only for the first case (table 2). Only one determination of 'intrinsic pigment' was made for each set and deductions at other concentrations were calculated. For each urine the experiments under the 4 sets of conditions were all performed on the same day.

Comparison of the results does not reveal any striking differences in pattern. The differences between the results with the same extract under the different conditions

TABLE 2. URINE EXTRACTS EXAMINED AT DIFFERENT TEMPERATURES AND CONCENTRATIONS OF ALCOHOL

Temperature	Concentration of alcohol	Aliquot	Treatment	D	2-log (100-D)	L_c	L_{fc}	Mg./specimen
°C.	%	cc.						
Case M.H. Age 20. Normal pregnancy 8 months; 3–12 hour urines. Total volume alcohol 100 cc.								
30	95	0.0	B	9.2	0.0419	—	—	—
		2.5	A	7.8	0.0353[1]	—	—	—
		2.5	B	39.7	0.2197	0.1778	0.1425	41.8
		5.0	B	57.4	0.3706	0.3287	0.2581	37.8
30	60	0.0	B	2.6	0.0114	—	—	—
		2.5	A	10.6	0.0487[1]	—	—	—
		2.5	B	31.4	0.1637	0.1523	0.1036	43.0
		5.0	B	50.5	0.3054	0.2940	0.1966	40.7
25	95	0.0	B	6.1	0.0273	—	—	—
		1.0	A	3.0	0.0132[1]	—	—	—
		1.0	B	19.4	0.0937	0.0664	0.0532	52.9
		1.5	B	24.9	0.1244	0.0971	0.0773	51.1
		2.5	B	34.5	0.1838	0.1565	0.1235	49.0
25	60	0.0	B	2.0	0.0088	—	—	—
		1.0	A	3.6	0.0159[1]	—	—	—
		1.0	B	14.0	0.0655	0.0567	0.0408	59.2
		1.5	B	19.3	0.0931	0.0843	0.0605	58.5
		2.5	B	28.7	0.1469	0.1381	0.0983	57.0

[1] The difference in "pigment" contribution here are mainly due to the relative insolubility of the pregnandiols present in the 60% alcohol runs.

are frequently larger than the error of determination of pure androsterone. These differences are due to the different behavior, with change of the 2 variables considered of the complex mixtures which make up urine extracts.

In this work use was made of either the Corning 401 or Rubicon 520 filter. Comparison using the same material of results obtained with these 2 filters was desirable. This was done with residual material from *case B.R.* (table 3).

Some of the residual material from *case B.R.* was also treated with Girard's reagent. The results are indicated in table 4. At the same time, an aliquot of the material was determined in the usual manner (total contribution). The greater part of the chromogens for the Zimmermann reaction is in the ketonic fraction. Deviations from Beer's law are present even in the ketonic fraction runs, though the concentration range is not as large as desired, due to lack of material. The pigment, practically linear in contribution, concentrated in the non-ketonic fraction as expected; some went into the aqueous phase, so the values are less than with the total. In table 4 we have also shown data on a ketonic fraction from the urine of *case J.W.* (also previously noted in

TABLE 2A. URINE EXTRACTS EXAMINED AT DIFFERENT TEMPERATURES AND CONCENTRATIONS OF ALCOHOL

Temperature	Concentration of alcohol	Aliquot	L_{fe}	Mg./specimen
		cc.		
Case J.M. Age 54 Male				
270 cc. urine				
Total volume alcohol 100 cc.				
30	95	0.0B	0.0535	—
		1.0B	0.0178	13.0
		2.5A	0.0241	—
		2.5B	0.0439	12.9
		5.0B	0.0814	11.9
30	60	0.0B	0.0177	—
		1.0B	0.0115	11.9
		2.5A	0.0200	—
		2.5B	0.0283	11.7
		5.0B	0.0581	12.1
25	95	0.0B	0.0348	—
		2.5A	0.0269	—
		2.5B	0.0336	13.3
		5.0B	0.0608	12.1
25	60	0.0B	0.0128	—
		2.5A	0.0237	—
		2.5B	0.0220	12.8
		5.0B	0.0433	12.6
Case J.W. Age 11 Female. Masculinizing syndrome. Butyl alcohol-soluble fraction of 2–24 hour urines.[2] Equivalent total volume of alcohol 500 cc.				
30	95	0.0B	0.0477	—
		5.0A	0.0092	—
		5.0B	0.0704	51.5
		10.0B	0.1339	49.0
30	60	0.0B	0.0168	—
		5.0A	0.0083	—
		5.0B	0.0465	48.2
		10.0B	0.0939	48.7
25	95	0.0B	0.0339	—
		5.0A	(0.0137)	—
		5.0B	0.0538	53.4
		10.0B	0.1082	53.7
25	60	0.0B	0.0110	—
		5.0A	0.0088	—
		5.0B	0.0402	58.4
		10.0B	0.0782	56.7
Case E.O.C. Age 33 Female				
24-hour urine				
Total volume alcohol 100 cc.				
30	95	0.0B	0.0526	—
		2.5A	0.0137	—
		2.5B	0.0776	22.8
		5.0B	0.1484	21.8
30	60	0.0B	0.0164	—
		2.5A	0.0141	—
		2.5B	0.0530	22.0
		5.0B	0.0993	20.6
25	95	0.0B	0.0367	—
		2.5A	0.0132	—
		2.5B	0.0636	25.3
		5.0B	0.1193	23.7
25	60	0.0B	0.0132	—
		2.5A	0.0137	—
		2.5B	0.0422	24.5
		5.0B	0.0861	25.0
Case J.W. Residual urine after butyl alcohol extraction, from 2–24 hour urines. Equivalent total volume alcohol 400 cc.				
30	95	0.0B	0.0526	—
		5.0A	0.0070	—
		5.0B	0.1085	63.6
30	60	0.0B	0.0141	—
		5.0A	0.0083	—
		5.0B	0.0723	60.0
25	95	0.0B	0.0381	—
		5.0A	0.0083	—
		5.0B	0.0728	57.8
		10.0B	0.1536	61.0
25	60	0.0B	0.0110	—
		5.0A	0.0088	—
		5.0B	0.0575	66.7
		10.0B	0.1189	69.0
Case B.R. Age 31 Female. Sterility				
3–12 hour urines.				
Total volume alcohol 100 cc.				
30	95	0.0B	0.0438	—
		2.5A	0.1046	—
		2.5B	0.2161	63.4
		5.0B	0.3910	57.4
30	60	0.0B	0.0128	—
		2.5A	0.1073	—
		2.5B	0.1515	62.8
		5.0B	0.2729	56.6
25	95	0.0B	0.0287	—
		1.0B	0.0792	78.7
		2.5A	0.1146	—
		2.5B	0.1710	68.0
		5.0B	0.3075	61.1
25	60	0.0B	0.0088	—
		1.0B	0.0568	82.5
		2.5A	0.1129	—
		2.5B	0.1270	73.7
		5.0B	0.2339	67.8

[2] Sodium pregnandiol glucuronide mostly removed.

table 2A). Deviation from linearity here is small probably because this would be a sample (from the nature of the case) containing a high proportion of 17-ketosteroids. Compare with Friedgood and Whidden (6).

In the paper by Neustadt there is no mention of any deviation from Beer's law in determining urine extracts. Since in the course of this work such deviations were noted we investigated the matter further. In the first two sets of table 5 the conditions were chosen to favor adherence to Beer's law; the temperature is about 25° C., and the effective concentration of alcohol in the reaction mixtures is about 82%, higher than that in the procedure of Friedgood and Whidden (6); the filter chosen passes a narrower band of wavelengths than the Corning 401 filter. Nevertheless, deviation is marked. As to the 'intrinsic' pigment in *case M.M.* the data for which are given in full, it may be noted by plotting that the pigment obeys Beer's law over the entire range of concentrations; in *case A.S.* there was a relatively small deviation, but it was positive in nature (fig. 1). In the last set of determinations we attempted the use of absolute alcohol. Trouble was experienced due to the appearance of car-

TABLE 3. COMPARISON OF RESULTS WITH TWO DIFFERENT FILTERS

Aliquot	L_{fc}	Mg./specimen	L_{fc}
cc.			
Case B.R. 30° C. 60% alcohol			
	Corning #401 filter		Rubicon #520 filter
1.0	0.0650	67.4	0.0741
2.5	0.1496	62.0	0.1574
5.0	0.2769	57.5	0.2705
2.5[1]	0.1024		0.1221

[1] "Pigment" values.

bonate, though the 15% alcoholic KOH was filtered and clear at the start. Here the order of the values is likewise the order of preparation, starting with clear KOH. In calculation, fortunately the fact that the *A series* of determinations were close in order to the corresponding *B* runs made it possible to correct for turbidity due to carbonate as well as intrinsic pigment. Despite the progressive turbidity, on plotting it was deduced that the pigment itself probably obeyed Beer's law. There is, within experimental error, which is here larger than usual, a region of apparent obedience to Beer's law in the result, but deviations do occur at higher concentrations. However, the total use of absolute alcohol or of 95% alcohol is not practical for the Oesting-Neustadt procedure. A set in which 95% alcohol was entirely used gave so much trouble because of carbonate that no conclusions could be drawn from it. Further the blanks are extremely high under these conditions.

In none of our experiments was Norite or other adsorbent used in the preparation of the urinary extracts; however Friedgood and Berman (7), using the method of Friedgood and Whidden observed deviations from Beer's law although their specimens had had this treatment; Saier and Grauer (16), using the Neustadt method for urine extracts found deviations at 25° C. though Norite is routinely used in their laboratory. We have felt that since the pigment values are linear or nearly so, the procedure of simple deduction of these values from the total color was justified. In the event however that some of the material contributing to the reading obtained for 'intrinsic pigment' should react with or be altered by the additional component m-dinitrobenzene the problem would become more complex.

Friedgood and Berman (7) felt that they could correct for deviations from Beer's law by drawing a straight line through their points and then subtracting the zero con-

centration intercept. It is possible to draw straight lines through our data also in many cases but the deviations from the mean thereby indicated do not in our opinion line up with the care with which the determinations were made and the possible precision of the apparatus. The drawing of smooth curves is possible with our data in great part and indeed with those of Friedgood and Berman if only the linear principle is abandoned. We have re-plotted some of their data and feel that there is a gradual

TABLE 4. RESULTS OBTAINED ON TREATMENT WITH GIRARD'S REAGENT

Aliquot	L_{fe}	Mg./specimen
cc.		
Case B.R. 30° C. 60% Alcohol—Corning #401 Filter		
	Total (Untreated)	
0.0B	0.0128	—
1.0A	0.0362	—
1.0B	0.0600	62.2
2.5A	0.0872	—
2.5B	0.1464	60.7
5.0A	0.1805	—
5.0B	0.2602	54.0
	Non-ketonic	
0.0B	0.0128	—
1.0B	0.0026	2.7
2.5A	0.0580	—
2.5B	0.0101	4.2
5.0A	0.1209	—
5.0B	0.0200	4.1
	Ketonic	
0.0B	0.0128	—
1.0A	0.0031	—
1.0B	0.0496	51.5
2.5A	0.0088	—
2.5B	0.1139	47.2
5.0B	0.2176	45.1
Aliquot	L_{fe}	Mg.[1]/sample
cc.		
Case J.W. Room Temp. 60% Alcohol		
Corning #401 Filter. Total volume 50 cc. alcohol		
0.0B	0.015	—
1.0A	0.000	—
1.0B	0.040	20.7
2.0A	0.001	—
2.0B	0.079	20.5
4.0A	0.001	—
4.0B	0.157	20.4
8.0A	0.004	—
8.0B	0.302	19.6

[1] Using K = 2.70 at 30°.

non-linear deviation from Beer's law. In working with such data, plotting is essential to observe general trends. If our data of table 5 be plotted (fig. 1) one may draw close-fitting straight lines by ignoring some of the points, or, by selecting parts of the range of concentrations we used, draw different straight lines with different o intercept for each. This point is not always obvious where only a small concentration range is employed. We should emphasize that we are conscious of the great difference in procedure between the Friedgood method and the one that we use; we mention the two in conjunction because the same problem has arisen in each and because our examination of their data suggests to us a different type of interpretation like that drawn from our own data.

In considering such clearly non-linear data as those of our first two sets of table 5, one may employ an empirical equation $L_{fe} = A + Bc + C\,c^2 + D\,c^3 \cdots$. Here A will be 0. If it is specified that C shall be a positive quantity, then $L_{fe} = B\,c - C\,c^2$, omit-

TABLE 5. DEVIATIONS FROM BEER'S LAW WITH WIDE VARIATION OF CONCENTRATION

Aliquot	2-log (100-D)	L_e	L_{fe}	Mg.[1]/specimen
cc.				
		25.7° C. 95% Alcohol, Rubicon 520 Filter		
Case M.M. Age 48, Male				
2–24 hour urine				
Total volume alcohol 100 cc.				
0.0B	0.0370	—	—	—
1.0A	0.0259	—	—	—
1.0B	0.1035	0.0665	0.0406	37.9
1.5A	0.0438	—	—	—
1.5B	0.1337	0.0967	0.0529	32.9
2.5A	0.0721	—	—	—
2.5B	0.1918	0.1548	0.0827	30.9
5.0A	0.1397	—	—	—
5.0B	0.3251	0.2881	0.1484	27.7
7.5A	0.2197	—	—	—
7.5B	0.4584	0.4214	0.2017	25.1
10.0A	0.2882	—	—	—
10.0B	0.5800	0.5430	0.2548	23.8
12.5A	0.3696	—	—	—
12.5B	0.6946	0.6576	0.2880	21.5

[1] Using an assumed value of K = 3.0.

Aliquot	L_{fe}	Mg.[1]/specimen	Aliquot	L_{fe}	"Mg."[2]/specimen
cc.			cc.		
		Rubicon 520 Filter			
25.7° C. 95% Alcohol			25.7° C. Absolute alcohol entirely		
Case A.S. Age 32, Female			*Case* M.B. Age 24, Female		
24-hour urine			24-hour urine		
Total volume alcohol 100 cc.			Total volume alcohol 100 cc.		
0.0B	0.0351	—	1.0	0.0636	6.36
1.0A	0.0092	—	1.5	0.0917	6.10
1.0B	0.0078	7.3	2.5	0.1623	6.50
1.5A	0.0159	—	5.0	0.3281	6.55
1.5B	0.0454	28.3	7.5	0.4555	6.08
2.5A	0.0273	—	10.0	0.5717	5.72
2.5B	0.0713	26.6	12.5	0.6539	5.23
5.0A	0.0595	—			
5.0B	0.1258	23.4			
8.0A	0.1007	—			
8.0B	0.1803	21.1			
12.0A	0.1586	—			
12.0B	0.2416	18.8			
16.0A	0.2132	—			
16.0B	0.3015	17.6			

[1] Using an assumed value of K = 3.0.

[2] $L_{fe} \times \frac{100}{cc.}$.

ting higher terms. This was done by Langstroth and Talbot (17) for androsterone. Here B c represents Beer's law contribution. The equation may perhaps be better written as $L_{fe} = B\,c - B\,D\,c^2$ or $B\,c(1 - D\,c)$. Then B gives the measure of the chromo-

genic potency of the solution and D is a term probably different for different mixtures involving the magnitude of the deviation. B is likewise the slope at o concentration, as may be shown by taking the first derivative. It is clearly indicated by the magni-

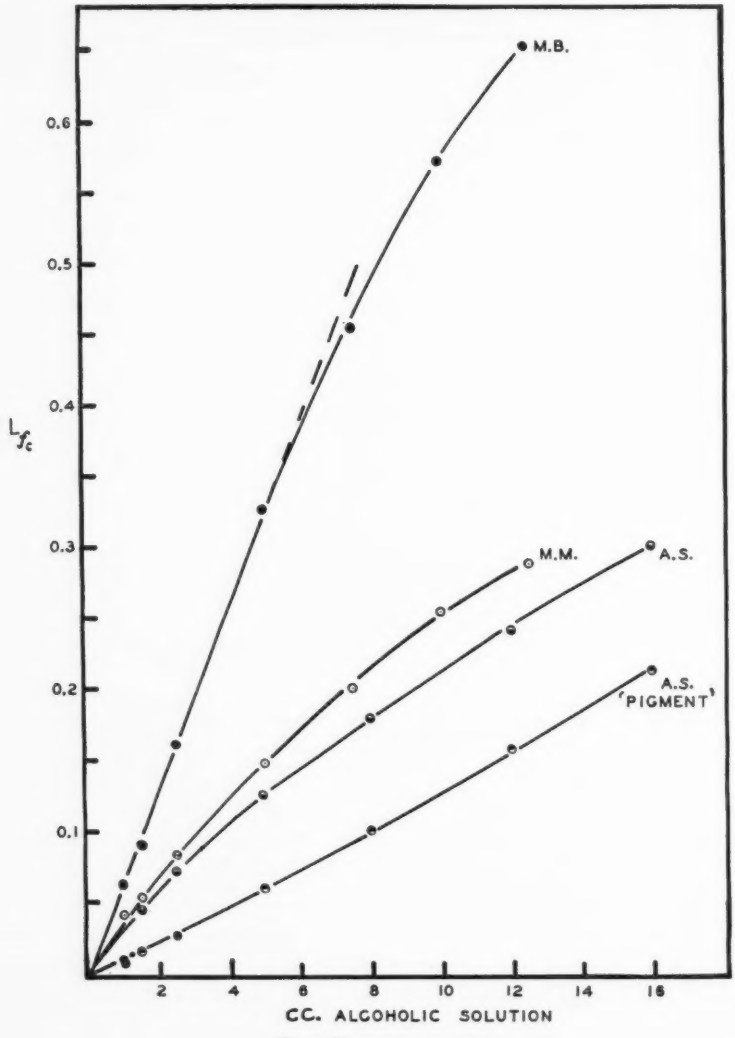

Fig. 1. Data from table 5.

tudes of the results we get that the Beer's law contribution, if given by B c, is much higher than values obtained by an absolute alcohol method such as that of Callow or Talbot.

Friedgood and Berman (7) also ran experiments on the same solutions by the

Talbot method. Their data by this method extend over the relative concentration range .05 cc. to .20 cc. only; their data by the Friedgood-Whidden method frequently extend over wider ranges; however if one examines these latter data over the relative range 1 to 4 only one still notes deviations from their tabulations of mg. of androsterone equivalents per cc., while their Talbot method data show no such deviations. The formulation developed above if applied to their data by the Friedgood-Whidden method would indicate results far above the values they obtained by the Talbot technic.

The Callows (13) and Langstroth and Talbot (17) observed, using absolute alcohol methods, that pure androsterone deviates from Beer's law at high concentrations.

TABLE 6. EFFECT OF DILUTION AFTER COLOR DEVELOPMENT

Aliquot	L_{fc}	Units[1]/specimen
cc.		
26.5° C. Rubicon 520 Filter 95% Alcohol. 2000 cc. pooled urine from four normal males, ages 21–32. Total volume alcohol 105 cc.		
0.0B (mean)	0.0614	—
1.0A	0.0209	—
1.0B	0.0520	47.8
2.0A	0.0410	—
2.0B	0.1010	46.4
4.0A	0.0830	—
4.0B	0.1780	40.9
6.0A	0.1226	—
6.0B	0.2549	39.0
8.0A	0.1612	—
8.0B	0.3180	36.6
12.0A	0.2343	—
12.0B	0.4418	33.8
16.0A	0.3072	—
16.0B	0.5486	31.5
1:1 Dilution with reagent blanks Original Concentration		
2.0	0.0562	51.6
4.0	0.1017	46.7
8.0	0.1780	40.9
16.0	0.3105	35.7

[1] Assuming K = 3.2.

It is not unreasonable to suggest that some other compounds found in neutral fractions of urine extracts may deviate from Beer's law at lower concentrations. However there may also be the type of interference shown by testosterone in mixtures with androsterone in the studies of Langstroth and Talbot (17). Whenever water is present not only will dehydroisoandrosterone give a higher color value than androsterone but interferences between various chromogens may be increased markedly in the concentrations customarily employed in such assay, thus resulting in the deviations noted.

Holtorff and Koch (18) in their method mention the change of intensity of developed color with time after dilution. The reason for this effect is evidently not known. We had a few data obtained by diluting solutions 1:1 immediately after reading and then reading again within 2 to 4 minutes. The first sets of runs on pure androsterone at 3 temperatures (table 1) were so diluted with 60% alcohol; K as computed from these results is higher than the data of table 1 by up to .5 unit. In

urine extract determinations we found that those intrinsic pigment solutions so diluted followed Beer's law. Chromogen tubes diluted with solvent or with solvent and KOH or with corresponding pigment tubes did not agree well after calculation with either the regular runs of that effective concentration or with one-half the original color contribution. Neustadt had recommended that if the solutions are too dark, one should dilute with alcohol and the reagents and read again. On one occasion we diluted a sample with the *B* reagent blank run concurrently; this agreed with the regular run equivalent in concentration (60% alcohol, C 401 filter used).

Later we followed this lead in one of the wide concentration experiments, the conditions being as for the first 2 sets of table 5. Extra *B* blank tubes were made up, to be used for dilution of the *B series* (chromogen) tubes next to them in chronological order of reading. These data are given in table 6. For each of the 4 dilutions made the result agreed well with that from ½ the concentration of the tube so diluted. Pigment contribution in this set was linear, save at the highest concentrations but the chromogen deviated from Beer's law as in the previous data.

These results appear to indicate that interferences in the absorption of light at the higher concentrations are primarily responsible for the progressive deviation from Beer's law which is observed. On this basis our selection of low concentrations for routine work[5] and for the experiments of table 2 is justified. From evidence previously cited evidently neither androsterone nor dehydroisoandrosterone nor mixtures of these (within certain limits) show such effect. Further, this effect appears to be minimized by the elimination of water from the reaction mixture.

The components of the urine extracts causing this phenomenon of deviation might arise from the effect of HCl on the steroids by ordinary hydrolysis (5, 19); however, it may be that relatively prolonged purification methods are also necessary after use of an effective system of simultaneous hydrolysis and extraction. From the data of table 4, it would appear that use of Girard's reagent with samples prepared in the older manner is not sufficient. It is indicated that a study of extracts, prepared so as to alter as little as possible the original steroids, and further purified by various of the recently proposed technics (20, 21, 22, 23) will be necessary to clarify the matter. It is not necessarily indicated that methods using reaction mixtures containing water are obsolete.

It is to be noted that results obtained by the system we have used for routine clinical studies are not comparable with other data reported in the literature. This is indeed the condition which must increasingly prevail, however, as better methods are more widely adopted.

SUMMARY

In the use of a standardized modification of the Oesting-Neustadt method for urinary androgens, both pure androsterone and various neutral fraction urinary extracts prepared in the usual manner showed increase in color developed on increasing temperature and concentration of alcohol. However the results with urinary extracts referred to androsterone as standard varied somewhat with different conditions as might be expected of a complex mixture of reactants. In these experiments deviations from Beer's law were indicated with change of concentration of the urinary extracts but not with pure androsterone or (from the work of Saier and Grauer) with dehydroisoandrosterone or mixtures of the 2. Such deviations were also indicated in 2 experiments when Girard's reagent was used. The existence of such deviations was confirmed by a series of experiments wherein the concentration of the neutral fraction

[5] We had arbitrarily made the rule that L_{fe} should not exceed 0.200 for routine work.

urinary material was varied over a wide range. Use of absolute alcohol in one experiment minimized the deviations; however use of absolute or 95% alcohol entirely is not practical for the Oesting-Neustadt method. Experiments wherein 1:1 dilution with reagent blanks were carried out after color development yielded results agreeing with those obtained directly from the corresponding concentration (½ the original). Such findings suggest that the deviations from Beer's law may be due to interferences and justify our current practice of using small amounts of urinary extract material for determination. It is suggested that extended preliminary purification as well as mild hydrolysis methods may yield material obeying Beer's law even when water is present in the reaction mixtures by eliminating the unknown interfering material producing the effect found.

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PITUITARY REPLACEMENT THERAPY IN PSEUDO-HYPOPHYSECTOMY

EFFECTS OF PITUITARY IMPLANTS UPON ORGAN WEIGHTS OF STARVED AND UNDERFED RATS

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IN PREVIOUS REPORTS (1–5), there was described a condition resembling hypophysectomy which was produced by underfeeding rats. The atrophy of the endocrine organs of the rat resulting from chronic inanition has been designated pseudo-hypophysectomy not only because of its resemblance to the effects of hypophysectomy but also because it is believed that many of the effects of inanition are due to malnutrition of the hypophysis, resulting in a diminished secretion of hormones. The endocrine tissue changes are qualitatively similar in the two conditions but they are seldom so marked during chronic inanition as they are after hypophysectomy. Changes in other tissues such as the spleen, thymus, and liver are equally marked in both conditions and appear to be related to the loss in body weight.

The purpose of the present study is to determine the effects of complete hormonal replacement therapy in the form of rat pituitary implants, in modifying the effects of complete starvation and of chronic inanition.

METHODS

Albino rats of the Sherman strain were used in these experiments. At the beginning of the experimental period the rats weighed approximately 200 gm. (males, 65 and females, 125 days of age). Ten male and 10 female fully fed rats were autopsied at the beginning of the experiment in order to obtain a control set of organ weights. It has been shown that such rats have fairly constant organ weights and respond in a uniform manner to inanition (3). The implant procedure was performed using sterile technic. A donor rat was decapitated and its hypophysis was dissected out and sucked into a trocar. The trocar was plunged into the leg muscle and a stilette was used to implant the gland into the muscle of the recipient. In most cases the control groups were implanted with a piece of brain equal in size to the pituitary gland in order to determine the effects of handling the animals, of trauma, and of possible infection. Fifty-five female and 10 male rats received either pituitary or brain implants.

At the termination of each experiment, the rats were lightly anesthetized with ether and exsanguinated from the heart. The organs were dissected rapidly and weighed on an analytical balance. The results are detailed in tables 1 and 2.

RESULTS

A. *Effects of Pituitary Implants in Normally Fed Rats*

Five rats received 6 to 8 implants of pituitary glands over an average period of 10 days. The results are shown in table 1, *group C*, and are to be compared with the nor-

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mal animals of *groups A* and *B*. There was the usual slight loss in body weight due to the handling and the injections. The thymus lost 50% of its weight, an effect which was probably mediated through the increase in estrogen produced by the hyperstimulated ovaries (6). The adrenal glands of the implanted rats were on the average no heavier than those of the untreated controls. The ovaries were markedly stimulated and upon gross examination showed numerous large follicles and recent corpora lutea. The largest pair of ovaries weighed 137 mg. as compared with the largest control pair of 73.8 mg. However, the combined weight of the uterus and vagina of the

TABLE 1. EFFECTS OF PITUITARY GLAND IMPLANTS IN FEMALE RATS[1]

Group	Days Starved or Underfed	Initial Body Wt. gm.	Final Body Wt. gm.	No. of Pituitary Implants	No. of Brain Tissue Implants	Organ Weights in milligrams								
						Hypophysis	Thyroids	Thymus	Liver	Kidneys	Spleen	Adrenals	Ovaries	Uterus and Vagina
Normally Fed														
A	0	199	199	0	0	12.0	17.2	224	6300	1480	620	46.8	53.2	600
B	0	198	198	0	0	11.9	15.1	340	6100	1470	940	43.7	63.7	470
C	0	200	190	7	0	10.4	15.8	150	6710	1470	750	44.7	82.2	560
Completely Starved														
D	8	192	118	7	0	10.9	12.7	17	3870	1300	200	76.7	74.8	590
E	8	191	115	0	7	8.7	10.2	44	3170	1120	190	55.3	38.0	390
F	8.4	199	116	6	0	10.5	12.1	40	3270	1110	220	66.3	68.2	640
G	9.2	199	114	0	7	7.7	9.8	49	3330	1020	270	46.1	37.3	290
Chronically Underfed														
H	152	204	141	8	0	6.9	13.3	58	4440	1190	450	38.6	33.2	300
I	151	198	148	0	0	6.8	13.9	83	3820	1030	370	30.0	24.9	250
J	113	197	128	10	0	8.4	11.4	28	4170	1150	320	48.3	32.6	350
K	114	194	129	0	10	6.9	14.4	53	4000	1060	350	33.9	16.0	230
Chronically Underfed and Ovariectomized														
L	227	199	136	22	0	7.3	13.1	60	4640	1160	450	40.2	(15.2)[2]	210
M	227	197	139	0	22	8.9	12.1	66	3970	1140	430	28.3	(15.2)[2]	210

[1] Figures are averages for 5 rats in each group. [2] Removed surgically before implantations were begun.

implant group was about the same as that of the untreated controls (an adaptation to estrogen? Selye (7). The rats were in estrus for from 2 to 3 days and then developed anestrus for the duration of the experiment.

B. Effects of Pituitary Implants in Starved and Chronically Underfed Rats

Effects upon the pituitary gland of the recipient. In completely starved rats, the pituitary implant groups (*D* and *F*) showed higher hypophyseal weights than the brain-injected controls. Grossly this increase in weight appeared to be in part due to congestion with blood especially upon the surface of the gland.

Two control groups of chronically underfed rats showed a marked reduction in the weight of the pituitary glands (table 1, *groups I* and *K*). Two similarly underfed groups received pituitary implants. *Group H* had pituitary glands no larger than the controls, while *group J* had pituitaries slightly larger.

Effect on the liver. There was an average increase of 0.37 gm. in those starved or underfed rats receiving pituitary implants as compared with the controls. These livers were heavier in almost each individual case as well (tables 1 and 2).

Effects on the thymus. The results of pituitary implants on this organ were similar whether given to starved or underfed rats and may, therefore, be considered together. The average thymic weight in the control groups was 58 mg. and this fell to 34 mg. in the groups which were implanted with pituitaries. However, in those underfed rats which were ovariectomized pituitary implants resulted in no significant reduction in the weight of the thymus. It may, therefore, be stated that the 24 mg. decrease which followed the pituitary implants was not due to the direct effects of the

pituitary hormones upon the thymus, but was probably due to the estrogen secreted by the stimulated ovaries. This is in agreement with the observation that injections of estrogen decrease the weight of the thymus of normal rats (6).

Effect on the kidney. Smith (8) reported that in hypophysectomized rats pituitary implants resulted in an increase in the weight of the kidney proportionate to the general body increase. As the result of the pituitary implants, the kidneys of the underfed and starved female rats weighed an average of 0.13 gm. more than the controls, although they were still 0.28 gm. lighter than the kidneys of the normally fed rat. However, in the normally fed, in the male underfed, and in the ovariectomized rats, the weight of the kidneys was not influenced by the pituitary implants. Since estrogen results in a reduction and testosterone in an increase in the weight of the kidney (9), and the pituitary implants resulted in the secretion of sex hormones, the failure of the pituitary implants to influence the weight of the kidneys of the normally fed rat or of the male underfed rat was contrary to expectations.

Effect on the thyroid glands. The thyroid glands of completely starved rats increased slightly in weight as the result of pituitary implants, when compared to brain tissue controls. As was true for the hypophysis of these rats, the increase in the weight of the thyroid may be ascribed in part to the congestion with blood which was present especially upon the surface. In the chronically underfed intact or ovariectomized female rat, 6 to 35 pituitary implants had little influence upon the weight of the thyroid glands. The thyroid glands of all the pituitary treated rats averaged 12.4 mg. in weight, as compared with 11.9 mg. for the controls. Evidently, the thyroid gland of the rat is refractory to stimulation by a moderate number of pituitary implants. On the other hand, Stephens (10) has shown that in the underfed guinea pig, anterior pituitary extract did result in an increase in the weight of the thyroid. In contrast to the effects upon the rat, his experiments show that during inanition, the thyroid gland of the guinea pig was markedly sensitive to pituitary stimulation.

Effect on the spleen. Perla (11) obtained an increase in the weight of the spleen in normal and in hypophysectomized rats as a result of injections of beef anterior pituitary extract and postulated the existence of a splenotropic hormone. However, in our experiments pituitary implants did not influence the weight of the spleen of the normally fed rats; they did not increase the weight of the atrophic spleen of chronic inanition, nor did they prevent the loss of weight of the spleen which occurs during starvation.

Effects on the reproductive tract. Seven daily implants of pituitary glands into normally fed rats resulted in a gain of 23.8 mg. in ovarian weight above the average control. In the completely starved rats, the ovarian weight was 33.9 mg. (90%) greater in the pituitary implanted groups than in the controls which received brain tissue. However, the greater weight of the ovaries of the pituitary implant groups must not be considered as due entirely to a gain from the starvation level of ovarian weight, because the implants were begun when the ovaries were normal in weight. That is, despite the starvation, the implants raised the ovarian weight from the normal of 58.4 mg. to 71.5 mg. Without the pituitary implants the ovaries would have weighed 33.9 mg. less than they did.

Marrian and Parkes (12) were the first to inject subcutaneously small quantities of a suspension of rat pituitary glands into underfed rats. They obtained ovulation and estrus and although no organ weights were reported, their results may be considered to be similar to those obtained from the implantation of whole pituitary glands. In chronically underfed rats which had received 8 to 10 pituitary implants, the weight of the ovaries increased 12.4 mg. (60%) above the controls despite the continued underfeeding.

The estrous cycles of the normally fed rats were upset by the pituitary implants. After the implants were begun, each of the 5 rats completed a normal cycle but as the number of implants increased (4 to 6), 4 of the rats became completely anestrous. The anestrous state persisted for 6 days in one of these rats and remained so to the tenth day of the experiment when all were autopsied. The rat which still showed signs of estrus after the sixth implant had entered the nucleated epithelium stage but marked "cornification" did not occur.

The completely starved rats responded to the implants differently than did either the normal or the chronically underfed animals. Within 3 days of the removal of food, the rats became anestrous. However, when daily implants were given, beginning with the first day of starvation, most of the rats were found in typical estrus on the fourth day. During the next 5 days there was found a mixed smear of nucleated and cornified epithelial cells and occasional leucocytes. Autopsy was performed on the eighth to the tenth days, at which time some of the rats were markedly athreptic. Despite the cachectic condition, the uterus and vagina of these rats weighed more than those of the normally fed rats which had received an equal number of pituitary implants. The combined weight of the uterus and vagina of one rat (after the expression of much uterine fluid) was 840 mg. as compared to the highest weight of 610 mg. obtained in a normally fed rat similarly treated.

Chronically underfed rats present an anestrous type of vaginal smear. When such rats are injected with pituitary glands, they show an almost normal smear. Thus, during 9 to 11 days, depending upon the number of implants (8 to 10), these rats had from 1 to 3 estrual cycles. The cycles were slightly irregular in rhythm.

In castrated chronically underfed rats, pituitary implants had no effect upon the weight of the uterus and vagina, despite the fact that the adrenal glands were much larger in this group than in its control. A completely anestrous smear was evident in both groups throughout the experimental period.

Effect on the adrenals. In a previous report it was shown that 8 or more pituitary implants injected into chronically underfed rats restored the atrophic adrenals to normal weight despite the continued underfeeding (4). Additional experiments reported here reveal that removal of the ovaries did not alter these results. The fact that the adrenals hypertrophied under the influence of hypophyseal stimulation in the absence of the ovaries showed that the adrenal enlargement which occurs in the intact underfed animals was not due to the estrogen (9) secreted by the stimulated ovaries. Our experiments also reveal that despite the injection of 8 to 10 pituitary glands into chronically underfed rats, the weight of the adrenal glands did not exceed that of the normally fed rats. This contrasts sharply with the effect of the implants in completely starved rats. In the latter, the adrenal glands increased in weight as the starvation was prolonged and the pituitary implants caused even greater hypertrophy in every case. In one rat the adrenal glands weighed 102 mg. as contrasted to the normal glands which seldom attain a weight of 55 mg.

The effects of pituitary implants in the male rats (table 2) were generally similar to those in the female animals. The thyroid glands of the experimental group weighed (9.6 to 13.7 mg.) slightly but probably insignificantly more than the controls (10.0 to 11.3 mg.). Pituitary implants had a marked indirect effect in lowering the weight of the thymus (20 to 40 mg.) when compared to the effect of the brain implants (40 to 80 mg.). The decreased thymic weight was probably due to the stimulatory effect of the implants upon the testes and the production of testosterone. The kidney weights of the pituitary-implanted group (1,180 to 1,440 mg.) averaged only 40 mg. higher than those of the controls (1,160 to 1,340 mg.) despite the presence of increased amounts of testosterone as judged by the accessory genital tissue stimulation.

The pituitary implants restored to normal the weight of the atrophic adrenal glands of underfed male rats. Palpation of the testes showed that during the 4-month period of underfeeding previous to the implants, little atrophy had occurred. This may be attributed to the fact that the fall in the body weight was not great, that the testes of occasional groups of rats are resistant to the effects of chronic inanition (5), and that the rats which had the more marked atrophy of the testes had been used for other experiments. The implantation of pituitary glands into such a group resulted in testes which weighed 26% more than those of the controls. Only 1 control pair of testes fell within the range of the pituitary-treated group. The interstitial tissue was ap-

TABLE 2. EFFECT OF PITUITARY GLAND IMPLANTS IN MALE RATS[1]

Group	Days Underfed	Initial Body Wt. gm.	Final Body Wt. gm.	No. of Pituitary Implants	No. of Brain Tissue Implants	Organ Weights, in milligrams											
						Hypophysis	Thyroids	Thymus	Liver	Kidneys	Spleen	Adrenals	Testes	Epididymis	Seminal Vesicles	Prostate	Rest of Genitalia
Normally Fed																	
N	0	199	199	0	0	6.9	18.4	396	6720	1490	686	28.1	2140	506	363	346	970
Chronically Underfed																	
O	149	203	139	13	0	5.0	12.4	28	4510	1250	380	29.6	2560	710	670	480	1010
P	149	199	143	0	13	5.0	10.9	61	4380	1210	430	21.6	2030	500	190	200	740

[1] Figures are averages for 10 rats in group *N* and 5 rats in groups *O* and *P*.

parently stimulated by the pituitary implants as evidenced by an increase over the control of 40% in epididymal weight, 250% in seminal vesicle weight, 140% in prostatic weight, and 25% in the weight of the remainder of the genital tract.

DISCUSSION

It has been stressed repeatedly that inanition results in depression of the anterior pituitary gland resulting in a condition of partial hypophysectomy. To this has been applied the term pseudo-hypophysectomy because of the resemblance to true hypophysectomy. On the other hand, it must be remembered that pseudo-hypophysectomy differs qualitatively from hypophysectomy in several respects. Mulinos and Pomerantz (5) have shown that chorionic gonadotropin is capable of stimulating the atrophic germinal epithelium of the testes of the chronically underfed rat but not that of the hypophysectomized rat (13). Comparable differences were found to obtain for the female rat (14). The pseudo-hypophysectomized animals differ from those that have had the pituitary removed surgically in that the former still have the pituitary gland present. Although it is true that in inanition the pituitary gland is markedly depressed there is still some activity present and such animals must not be considered as being completely hypophysectomized.

The experiments performed by us differ from those of Smith (8) in that during chronic inanition glandular therapy must make itself manifest despite the continued undernutrition. The hypophysectomized animal loses in weight somewhat similarly to our animals. However, when food is allowed ad libitum and pituitary replacement therapy is instituted in these animals, there are manifest not only the effects from the specific gland therapy but also the restorative effects of the refeeding and the gain in body weight. The proper control with the two types of experiments would be to keep the hypophysectomized animals at the weight level at which they are at the beginning of the specific glandular treatment in order in this way to avoid the masking effects of the improved nutritional state.

In the present report it has been demonstrated that the responses to the im-

plantation of pituitary glands are due both to the specific hormonal effects upon the various glands and also to the secretions of these glands upon other organs. Since it is known that the sex hormones will affect the size of the thymus, the adrenal cortex and the kidney, one must distinguish between the indirect effects of the sex hormones and the direct affects of the pituitary implants upon these organs. Lastly, our experiments indicate that certain organs of these animals may become hypersensitive to the various hormones during starvation or chronic underfeeding. Thus, during starvation the adrenal glands enlarge and by means of injections of pituitary glands this enlargement may be further augmented to a size far beyond any that has been obtained in the normal animals.

SUMMARY

A condition of pseudo-hypophysectomy was induced in male and female rats by chronic underfeeding. These rats were implanted with rat pituitary glands for varying periods of time and the effects compared to the changes produced by pituitary implants in normal and completely starved rats. The pituitary implants decreased the weight of the thymus gland. This was mediated indirectly through the sex hormones since after castration pituitary implants did not result in thymic atrophy. The weight of the thyroid glands was unaffected and confirms the fact that it is difficult to stimulate the thyroid gland of the rat. There was no increase in the weight of the spleen thus adducing no evidence for the presence of any marked amount of splenotropic hormone in this number of pituitary implants.

Pituitary implants resulted in an increase in the size of the ovaries and testes. The accessory genital organs were stimulated. The estrual cycles of the normal rats were supplanted by anestrus. In the underfed rats, the anestrous smear was replaced by the cyclical type found in the normal state, and in the starved animals the implants resulted in signs of continuous estrus.

Seven pituitary implants resulted in no change in the weight of the adrenal glands of the normally fed rats. In the chronically underfed rat receiving a similar or greater number of implants, the adrenal glands returned to normal weight despite the continued underfeeding. In the female rats this phenomenon occurred in the absence of the ovaries as well, while in males it occurred in the presence of stimulated testes.

Starvation resulted in an increase in the weight of the adrenal glands. Pituitary implants injected daily into rats from the first day of starvation resulted in still further enlargement of the adrenal glands. Since starvation was the only state in which pituitary implants resulted in an enlargement of the adrenal glands to above the normal weight, it is suggested that the adrenal glands of starved rats may be hypersensitive to the adrenotropic hormone of the pituitary.

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CHEMICAL DETERMINATIONS OF THE ADRENO-CORTICAL (AC) HORMONES IN THE HEART MUSCLE OF THE RAT[1]

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THE IMPORTANCE OF THE MEDULLARY and cortical hormones of the adrenal in the function of the heart has been observed clinically and experimentally. A method has recently been devised by which it is possible to study the concentration of these hormones in the heart muscle under a variety of conditions (1, 2). The technic is based on a colorimetric method for determining the amount of adreno-cortical compounds present per gram of tissue. These compounds consist of sterols bound to adrenalin, in addition to 'free' adrenalin which is apparently not combined with the sterols. Ascorbic acid is likewise determined in these measurements. It has been reported that ascorbic acid forms 9 to 24% of the total adreno-cortical content of the heart (3).

The specificity ratio (4) which indicates the relationship between the adreno-cortical constituents and the ascorbic acid has been described (1, 2) and its significance may be approximately characterized by the following examples.

Specific ratios of.

1:0.3 = pure ascorbic acid. This specific ratio has not been found in blood or tissues.
1:0.7 = adreno-cortical hormones with a relatively high portion of ascorbic acid.
1:1.0 = adreno-cortical hormones with a small portion of ascorbic acid and little or no 'free' adrenalin.
1:1.8 = adreno-cortical hormones with a relatively large portion of 'free' adrenalin.
1:3.5 = pure adrenalin. This ratio was found in the heart muscle under some unusual conditions.

Specific ratios between 1:1.0 and 1:3.5 indicate an increase in the adrenalin content, in comparison to the sterols and ascorbic acid present.

The abbreviation AC is used for the total reading which is expressed in color units per gm. of tissue. One color unit (C.U.) corresponds to the color intensity of 1 mγ of adrenalin (1 billionth of a gram).

MATERIAL

The values established in this study were obtained from male albino and gray rats 2 to 3 months of age which weighed 150 to 200 gm. The heart weight is given in terms of the percentage of the total body weight.

Racial, seasonal and individual variations in terms of C.U. were determined for the adreno-cortical hormone content of the heart muscle of the animals. Subsequently determinations were made in animals following adrenalectomy, castration, injection

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TABLE 1. CONTROLS

White rats (summer)					White rats (winter)					Gray rats (summer)				
		Heart					Heart					Heart		
Rat No.	Day	% of body wt.	AC c.u./gm.[1]	sp.r. 1:[2]	Rat No.	Day	% of body wt.	AC c.u./gm.[1]	sp.r. 1:[2]	Rat No.	Day	% of body wt.	AC c.u./gm.[1]	sp.r. 1:[2]
1	7/26	0.32	274	0.60	21	12/5	0.31	1137	1.11	176	7/9	0.25	1084	—
2	7/26	0.35	130	0.80	22	12/5	0.30	1499	1.05	177	7/9	0.21	1090	—
3	7/29	0.31	237	0.50	23	12/7	0.31	1297	1.00	67	7/10	0.23	1102	—
4	7/30	0.31	589	1.20	24	12/7	0.30	1328	1.05	68	7/10	0.25	905	1.00
5	7/31	0.33	268	1.00	25	12/9	0.28	1078	1.21	69	7/11	0.26	1384	1.02
6	8/1	0.28	387	0.41	26	12/6	0.34	1218	1.35	70	7/11	0.25	1504	1.04
7	8/2	0.37	519	0.56	27	2/1	0.33	1205	1.06	71	7/12	0.28	1226	1.02
8	8/3	0.33	801	0.59	28	1/31	0.31	1284	1.02	72	7/12	0.31	1091	1.31
9	8/5	0.37	424	0.63	29	2/1	0.28	1374	1.45	73	8/7	0.26	1352	0.73
10	8/6	0.38	878	0.76	30	2/2	0.32	660	1.38	74	8/8	0.32	1068	0.70
Average		0.33	451	0.71			0.31	1208	1.17			0.25	1180	0.97

[1] Color units per gram of tissue.
[2] Specificity ratio.

of adrenalin, desoxycorticosterone, insulin, thyroxin, testosterone propionate, and after exercise, exposure to cold, and treatment with digitalis glucosides.

RESULTS

Seasonal, racial and individual variations (table 1). The AC content of the heart muscle was higher in the winter than in the summer months. The average specific ratio for the summer months was 1:0.7, which would indicate a higher portion of ascorbic acid than in the winter.

The heart tissue of albino rats had a lower concentration of AC than that of gray rats. The ascorbic acid content was more conspicuous in the white rats.

There was a wide range of individual variation in the AC values and their specificity ratios in the different groups, but characteristic averages for the groups were established.

TABLE 2. ADRENALIN INJECTION

White rats						Gray rats					
			Heart						Heart		
Rat No.	Day	Min. of survival[1]	% of body wt.	AC c.u./gm.	sp. r. 1:	Rat No.	Day	Min. of survival[1]	% of body wt.	AC c.u./gm.	sp. r. 1:
41	7/25	20	0.33	650	0.80	75	7/15	12	0.38	1928	1.05
42	7/25	20	0.34	1439	2.53	76	7/16	20	0.37	1242	1.18
43	7/29	20	0.36	1568	1.73	77	7/16	20	0.30	1694	1.55
44	7/30	20	0.44	—	—	78	8/7	20	0.33	1636	1.66
45	7/31	20	0.35	784	2.50	79	8/8	20	0.30	1619	1.48
46	8/1	20	0.29	1059	1.11	80	8/8	7	0.31	4773	1.18
47	8/2	12	0.40	1958	1.11	81	8/9	9	0.30	2654	0.96
48	8/3	20	0.33	1172	1.52	82	8/9	10	0.37	3380	1.10
49	8/5	20	0.31	920	3.07	83	8/10	5	0.32	3374	1.66
50	8/6	20	0.34	1467	1.37	84	8/10	5	0.34	1932	1.32
Average		20	0.35	1224	1.81			13	0.33	2423	1.31
Deviation from summer standard			+0.02	+171%					+0.08	+106%	

[1] All deaths which occurred earlier than 20 minutes after the injection of adrenalin were spontaneous. In gray rats 80–83 the effect of the injected adrenalin had been speeded up by massage of the area of the injection. *Rats 41–50* and *75–77* received 2.5γ and *rats 78–84* 5.0γ of adrenalin per gram of body weight.

TABLE 3. DESOXYCORTICOSTERONE ACETATE WITHOUT AND WITH ADRENALIN

Cortate i.m.[1]					Cortate i.m.[1]+Adrenalin s.c.[2]					
Rat No.	Day	Heart			Rat No.	Day	Min. of survival	Heart		
		% of body wt.	AC c.u./gm.	sp.r. 1:				% of body wt.	AC c.u./gm.	sp.r 1:
57	8/15	0.33	1394	0.95	178	8/15	20	0.33	1136	1.22
58	8/16	0.35	796	—	179	8/16	20	0.33	1518	2.27
59	8/17	0.30	1146	0.96	180	8/17	20	0.32	1613	1.85
60	8/19	0.32	613	0.89	181	8/19	4[3]	0.36	1728	1.82
61	8/20	0.32	611	0.67	182	8/20	20	0.33	1353	1.53
62	8/22	0.37	1331	0.74	183	8/26	20	0.33	1081	2.37
63	8/23	0.32	1104	0.89	—	—	—	—	—	—
64	8/24	0.31	952	0.88	—	—	—	—	—	—
65	8/24	0.29	1237	1.84	—	—	—	—	—	—
66	8/26	0.28	845	0.64	—	—	—	—	—	—
Average		0.32	1003	0.94			18	0.33	1405	1.84
Deviation from summer standard		−0.01	+122%					±0.00	+211%	

[1] All animals were given a daily dose of 2.5γ/gm. body weight of desoxycorticosterone acetate for 8 days, a total of 20γ per gm.

[2] The animals of the second group received in addition 2.5γ/gm. of adrenalin the day following the last desoxycorticosterone acetate injection.

[3] *Rat 181* died spontaneously 4 minutes after the injection of adrenalin. The others were killed.

Adrenalin treatment (table 2). After adrenalin treatment there was an increase in the AC concentration in the heart muscle toward the side of 'free' adrenalin. The specific ratios were 1:1.81 in the albino rats and 1:1.31 in the gray rats. Seven animals (*47, 75, 80–84*) which died spontaneously showed evidence of pulmonary edema and dilatation of the heart. The AC content in these animals had reached a concentration of more than 1900 c.u. per gm. of tissue.

The weight of the heart muscle was increased after adrenalin administration.

Desoxycorticosterone acetate injection, with and without adrenalin (table 3). There

TABLE 4. ADRENALECTOMY[1]

Rat No.	Day of adrenalectomy	Day of castration	Survival after adrenalectomy, days	Heart		
				% of body wt.[2]	AC c.u./gm.	sp. r. 1:
85	11/12	—	5	0.33	319	1.00
86	1/18	—	5	0.26	816	1.33
87	1/24	—	6	0.35	1374	1.05
88	1/14	1/22	8	0.31	1062	1.56
89	1/28	—	10	0.23	762	—
90	1/29	—	11	0.22	1377	1.41
91	11/13	—	12	0.25	816	1.33
92	1/14	1/22	16	0.19	1196	—
93	11/30	1/22	54	0.25	1121	1.30
Average			14	0.26	937	1.28
Deviation from winter standard				−0.05	−22%	

[1] In the adrenalectomized rats of this series no remaining cortical tissue was found at autopsy. In *rat 87* there was a purulent infection of the left kidney. All animals had died spontaneously.

[2] All heart-body weight percentages were calculated with reference to the body weight before adrenalectomy. All rats lost some weight (on an average 22%).

TABLE 5. INSULIN INJECTION

Rat No.	Day	Insulin	Survival[1]	Heart		
				% of body wt.	c.u./gm.	sp.r. 1:
		U	*minutes*			
106	8/27	6	40	0.33	1125	—
107	8/27	5	120	0.31	1627	1.22
108	8/28	8	120	0.37	732	0.50
109	8/28	12	150	0.32	1149	0.88
110	8/30	8½	60	0.36	1255	1.38
111	8/30	6½	80	0.38	808	1.07
112	8/31	15	180	0.28	990	0.71
113	8/31	18	200	0.30	973	1.31
114	9/1	4	40	0.30	329	0.61
115	9/1	3	80	0.29	766	2.15
Average		8½	107	0.32	975	0.98
Deviation from summer standard				−0.01	+116%	

[1] All animals were killed. None of them had convulsions, some were drowsy.

was an increase in the AC concentration in the heart, and a specific ratio similar to that found with synthetic AC (1), after 8 days of intramuscular injections of desoxycorticosterone acetate.[2] If adrenalin was injected after a pretreatment with desoxycor-

TABLE 6. THYROXIN WITHOUT AND WITH ADRENALIN[1]

Thyroxin					Thyroxin plus adrenalin					
		Heart					Heart			
Rat No.	Day	% of body wt.[2]	AC c.u./gm.	sp.r. 1:	Rat No.	Day	Minutes of survival after adrenalin inj.[3]	% of body wt.	AC c.u./gm.	sp.r. 1:
116	9/17	0.42	668	0.93	184	11/5	3	0.43	3077	1.13
117	9/17	0.41	234	3.88	185	11/5	4	0.38	2721	1.12
118	9/18	0.46	597	0.71	186	11/5	4	0.40	2657	1.38
119	9/18	0.44	405	3.40	187	11/7	3	0.38	2207	1.21
120	9/19	0.38	499	0.66	188	11/7	5	0.44	2615	1.04
121	9/19	0.40	495	1.09	189	11/7	5	0.42	2228	1.11
122	9/20	0.41	—	—	—	—	—	—	—	—
123	9/20	0.42	351	3.80	—	—	—	—	—	—
124	9/21	0.41	511	1.50	—	—	—	—	—	—
125	9/21	0.41	706	1.53	—	—	—	—	—	—
Average		0.42	496	1.94			4	0.41	2584	1.32
Deviation from summer standard		+0.09	+10%		Deviation from fall standard[4]			+0.09	+211%	

[1] All animals had received a daily dose of 1 mg. of thyroxin for 3 days previous to the analysis. All animals of the second group received 2.5γ/gm. body weight of adrenalin 1 day after the last thyroxin.

[2] All heart-body weight percentages were calculated with reference to the body weight before administration of thyroxin.

[3] All animals of this group died spontaneously.

[4] The "fall standard" value of 829 c.u./gm. was calculated arbitrarily as the arithmetical medium between the 'summer' and the 'winter' values.

[2] Cortate, Schering Corporation, New Brunswick, N. J.

ticosterone acetate, the readings showed a specific ratio which would indicate the presence of 'free' adrenalin.

One rat (181) died spontaneously when the AC concentration was 1728 c.u. per gm. This was slightly less than the level observed in the animals which had died after receiving adrenalin only.

There was no significant change in the weight of the heart in either of the groups of experimental animals.

Adrenalectomy (table 4). Nine rats died several days after complete adrenalectomy. They showed a decreased level of AC which appeared to have taken place at the expense of the sterol component. Castration superimposed upon the adrenalectomy in some animals did not appear to affect the AC content of the heart.[3]

TABLE 7. TESTOSTERONE PROPIONATE INJECTION AND CASTRATION

Testosterone Propionate[1]					Castration					
Rat No.	Day	Heart			Rat No.	Day of castration	Days after castration	Heart		
		% of body wt.[2]	AC c.u./gm.	sp.r. 1:				% of body wt.[2]	AC c.u./gm.	sp.r. 1:
94	2/18	0.35	1103	1.16	100	2/14	10	0.29	1680	1.73
95	2/18	0.33	1459	1.62	101	2/14	10	0.36	1609	1.73
96	2/19	0.34	873	1.70	102	2/21	14	0.31	1312	2.98
97	2/19	0.43	1377	1.28	103	2/21	14	0.32	1290	2.12
98	2/20	0.35	953	1.33	104	2/22	15	0.34	732	1.25
99	2/20	0.37	1292	1.27	105	2/22	15	0.33	1294	1.77
Average		0.36	1176	1.39			13	0.32	1303	1.93
Deviation from winter standard		+0.05	−3%					+0.01	+8%	

[1] All animals of this group received a daily dose of 1 mg. of testosterone propionate i.m. for 8 days prior to analysis.

[2] All heart-body weight percentages were calculated with reference to the body weight before administration of testosterone propionate and castration respectively.

There was a decrease in the average of the weight of the heart after adrenalectomy.

Insulin injection (table 5). Comparatively large doses of insulin given subcutaneously produced an increase in the AC content of the heart muscle. The specific ratios were not indicative of 'free' adrenalin. The weight of the heart remained practically unchanged.

Thyroxin with and without the injection of adrenalin (table 6). Treatment with 1 mg. thyroxin daily for 3 days altered the AC content of the heart slightly with an increase in the 'free' adrenalin (specific ratio 1:1.94.). The heart was enlarged and abnormally heavy in all instances.

Adrenalin was administered in the same dosage as for the albino rats in table 2, on the 3rd day of thyroxin treatment. Death occurred immediately in all animals. The AC concentrations were more than 1900 c.u. per gm. of heart tissue. The relative and the absolute increase in the AC content was greater than after equivalent dosages of adrenalin alone. The specific ratios indicated relatively large amounts of sterols.

The administration of adrenalin did not change the enlargement of the heart produced by the action of thyroxin.

[3] In 3 instances the hearts were examined immediately after death; in other instances the dead animals had been kept for several hours in the refrigerator. This apparently did not produce any alteration in the results obtained, according to the control determinations.

TABLE 8. PHYSICAL EXERCISE[1]

Rat No.	Day	Min. of exercise	Heart			Rat No.	Day	Min. of exercise	Heart			Rat No.[2]	Day	Min. of exercise	Heart		
			% of body wt.	AC c.u./gm.	sp.r. 1:				% of body wt.	AC c.u./gm.	sp.r. 1:				% of body wt.	AC c.u./gm.	sp.r. 1:
146	9/11	6	0.33	1037	1.67	156	9/7	45	0.33	188	1.66	166	9/10	260	0.32	533	1.67
147	9/13	8	0.32	1034	2.20	157	9/9	50	0.34	502	3.10	167	9/13	300	0.34	1047	1.43
148	9/11	10	0.31	832	3.82	158	9/9	80	0.29	793	2.00	168	9/10	310	0.33	779	0.77
149	10/15	10	0.33	755	1.14	159	9/9	120	0.31	802	1.34	169	9/13	320	0.33	1197	2.48
150	10/15	10	0.31	942	1.74	160	9/10	180	0.31	615	2.53	170	9/13	400	0.32	536	2.93
151	10/15	10	0.32	879	1.58	161	9/10	210	0.33	558	1.95	171	9/13	420	0.35	1016	1.32
152	10/15	10	0.35	671	0.70	162	10/26	120	0.33	1369	1.23	172	10/25	500	0.31	1083	1.15
153	9/1	15	0.32	789	1.46	163	10/26	135	0.34	1348	1.40	173	10/25	520	0.29	511	4.00
154	9/9	20	0.30	921	0.87	164	10/26	150	0.31	1355	1.53	174	10/25	540	0.34	786	1.47
155	9/7	15	0.31	928	1.88	165	10/26	160	0.31	1073	1.22	175	10/25	560	0.30	519	0.77
Average		11	0.32	879	1.71			125	0.32	860	1.80			410	0.32	801	1.80
Deviation from summer standard			−0.01	+95%					−0.01	+91%					−0.01	+77%	

[1] All animals were forced to swim constantly in a large basin filled with lukewarm water.
[2] All animals of this latter group showed symptoms of fatigue and exhaustion to a minor or major degree.

TABLE 9. EXPOSURE TO COLD[1]

Rat No.	Day	Heart		
		% of body wt.	AC c.u./gm.	sp.r. 1:
126	9/22	0.31	976	1.56
127	9/22	0.27	871	1.96
128	9/22	0.27	929	1.75
129	9/23	0.29	463	1.27
130	9/23	0.32	1086	1.98
131	9/23	0.29	776	3.00
132	9/23	0.30	1134	1.30
133	9/24	0.32	792	1.39
134	9/24	0.28	296	4.12
135	9/24	0.32	642	3.60
Average		0.30	797	2.19
Deviation from summer standard		−0.03	+77%	

[1] All animals of this group were placed for 10 minutes in water of 5° C., and then killed.

Testosterone propionate injections and castration (table 7). The injection of a male gonadal sterol preparation[4] did not influence the AC content of the heart muscle. Castration did not produce any change in the total AC, but there was more 'free' adrenalin as indicated by the specific ratio of 1:1.93. The weight of the heart was increased after testosterone propionate. It was not affected by castration.

Physical exercise (table 8). Thirty rats were forced to swim in deep water. They were subdivided into 3 groups on the basis of the duration of the exercise which ranged from 6 to 560 minutes. In all groups of animals after exercise there was an increase in the concentration of the AC, with an increase in the 'free' adrenalin content. The average AC content was slightly less in the group of animals forced to exercise for the longest period of time. There was no significant change in the weight of the heart in these groups of animals.

Exposure to cold (table 9). Exposure to cold water (5° C. for 10 minutes) produced

TABLE 10. OUABAIN AND STROPHANTHIN[1] INJECTION

Rat No.	Day	Drug	Dose, mg.	Min. of survival	Heart		
					% of body wt.	AC c.u./gm.	sp.r. 1:
190	9/4	Ouab.	0.45	30	0.30	930	1.08
191	9/4	Ouab.	0.75	30	0.32	1178	1.00
192	9/5	Stro.	0.66	20	0.29	1274	1.87
193	9/5	Stro.	0.78	25	0.30	849	1.64
194	9/5	Stro.	0.60	30	0.32	673	2.00
195	9/5	Stro.	0.60	40	0.33	545	2.80
196	9/14	Stro.	2.50	30	0.33	933	3.47
197	9/14	Stro.	2.50	40	0.33	841	2.15
198	9/14	Stro.	2.50	50	0.36	765	1.62
199	9/14	Stro.	2.50	60	0.29	841	1.64
Average			1.38	36	0.32	883	1.93
Deviation from summer standard					−0.01	+96%	

[1] The preparations were injected intramuscularly. *Rats 196–199* (largest dose) showed ataxia and convulsions but none died spontaneously.

[4] Testosterone propionate in the form of Perandren, Ciba Pharmaceutical Products, Inc., Summit, N. J.

an increase in the AC and 'free' adrenalin content of the heart, with a specific ratio of 1:2.19. These changes were similar to those observed after physical exercise. There was a moderate decrease in the weight of the heart.

Digitalis glucoside injections (*Ouabian, Strophanthin K*) (table 10). The injection of toxic but not lethal doses of digitalis glucosides was followed by an increase in the AC and 'free' adrenalin content of the heart muscle. There was no change in the weight of the heart.

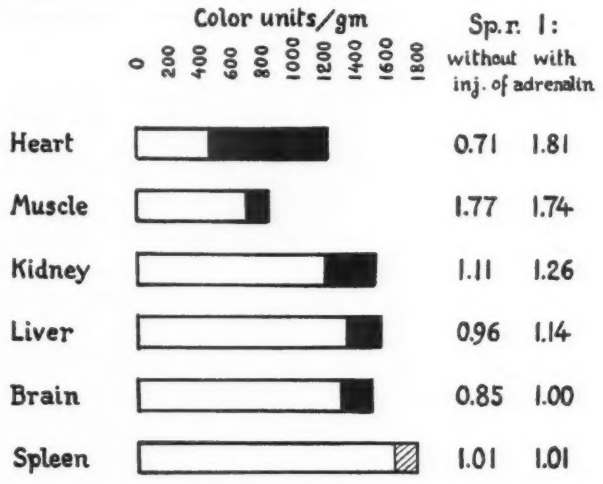

Fig. 1. Absolute AC content of organs of white rats (summer standards) without and after injection of adrenalin (2.5γ per gm. of body weight). The black areas indicate the increase of AC after adrenalin injection. The shaded area (spleen) indicates a slight decrease.

DISCUSSION

The heart muscle of the control albino rats was found to contain a smaller concentration of AC (adreno-cortical compounds), plus 'free' adrenalin and ascorbic acid than the other organs examined from the same animals (reference 2, fig. 1). The tissues examined were skeletal muscle, kidney, liver, brain and spleen. The values for skeletal muscle more nearly approximated those for the heart. The heart muscle absorbed more of the injected adrenalin than the other tissues examined (fig. 1). Desoxycorticosterone acetate was absorbed in greater quantity by the heart than by the kidney or liver.

Local adrenalin formation may account for the small deficiency of AC in the heart following adrenalectomy (5); replenishment from the AC stores of the spleen may also have been partially responsible (2).

The gonadal sterols did not appear to participate in the maintenance of the cardiac AC concentration. In normal animals the injected hormones of the male gonads did not modify the AC content, although castration did produce a slight increase in the 'free' adrenalin in the heart.

Thyroxin increased the 'free' adrenalin and appeared to enhance the absorption of injected adrenalin in the heart muscle.

Insulin with its stimulating effect upon the adrenal glands increased the content of AC in the heart muscle apparently through the deposition of the adrenalin-sterol compounds. This may be correlated with the increase of AC in the other organs of the rat (2) and in human blood (6) after the injection of insulin.

Physical exercise and exposure to cold, conditions known to increase physiologically the discharge of adrenalin from the adrenal glands (7), is accompanied by an accumulation of AC in the heart with a relatively large proportion of 'free' adrenalin. During physical exercise the AC content of the skeletal muscle was gradually depleted, and reached its lowest level with the appearance of fatigue symptoms (2). A similar type of storage and depletion of AC in the heart and skeletal muscles was observed after the administration of thyroxin (2).

Toxic doses of digitalis glucosides increased the adrenalin content of the heart. Some biologic relationships between the digitalis glucosides and the adrenal glands have been pointed out (8, 9).

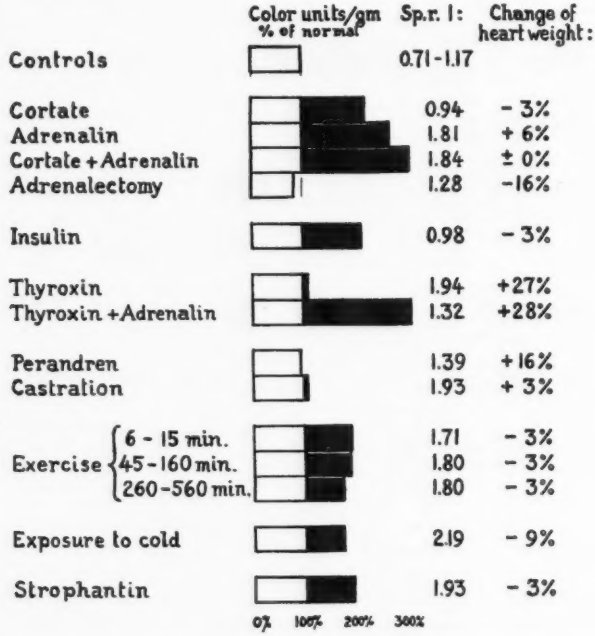

Fig. 2. Survey of changes of heart AC (% of normal standards), specific ratios and heart weights (the latter in % of absolute weights) under various experimental conditions.

Animals which died spontaneously had an AC concentration greater than 1900 c.u. (table 2, *rats* 47, 75, 80–84, table 6, *rats* 184–189). *Rat* 181 had a lower AC level at the time of death. This animal had been pretreated with desoxycorticosterone acetate and then received adrenalin (1728 c.u.).

The sensitization by thyroxin to adrenalin (10–12) was confirmed. Sixteen % of the rats given 2.5 γ of adrenalin per gm. of body weight and no thyroxin died spontaneously. All of the rats given the same dose of adrenalin after treatment with thyroxin died within 3 to 5 minutes. In all of these animals there was a high content of adrenalin in the heart muscle. The animals which died spontaneously after receiving adrenalin showed symptoms of acute heart failure as the cause of death.

Seasonal and racial variations of the AC content of the heart included higher AC values during the winter months, and in the dark or pigmented animals. This type of animal has larger adrenal glands than non-pigmented animals (13, 14).

Changes in the weight of the heart were observed in several groups of experimental animals. After 3 days of thyroxin treatment the heart weight was increased 27 to 28%, a confirmatory finding (15). There was a 16% weight increase after treatment with testosterone propionate, and no weight change after administration of desoxycorticosterone acetate. Both of these substances have been described as apt to produce arterial hypertension in the rat (16). After adrenalectomy there was a 16% decrease in the weight of the heart, likewise a confirmatory finding (17–19). Exposure to cold appeared to diminish the weight of the heart.

It may be justified to attach some significance in pathologic cardiac conditions to physiologic and toxic changes in the concentrations within the heart muscle of the adreno-cortical or AC hormones, since they have been shown to be of fundamental importance in metabolic and trophic cell functions (20–22). In patients with angina pectoris the AC content of the blood during physical exercise was high (23). The AC content of the blood was also high in patients with renal insufficiency and cardiac failure, and in those with pulmonary edema (24). Abnormalities of the AC content of the heart muscle of patients who died of cardiac failure have been found.

SUMMARY

Quantitative determinations of the concentration of the adrenal hormones (adrenalin and cortical sterols, 'AC') in the heart muscle of rats were made. Seasonal and racial variations were established in the AC content of the heart muscle. The AC level was increased after the administration of adrenalin and of desoxycorticosterone acetate. Increases of the AC content of the heart muscle were also observed following physical exercise, exposure to cold and the injection of insulin and strophanthin. The AC content of the heart muscle was diminished after adrenalectomy. Pretreatment with thyroxin enhanced the deposition of injected adrenalin in the heart muscle. The administration of testosterone propionate and castration had little effect. Death occurred in all animals with a myocardial content of AC which was greater than 1900 c.u. per gm. of muscle, with the signs of acute heart failure. The 'specificity ratio' proved a fairly reliable means for the differentiation of the mutual relationship between the adreno-cortical constituents.

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THE ATTENUATION OF INSULIN BY ADSORPTION[1]

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THE MANNER IN WHICH THE PHYSICAL PROPERTIES and physiological behavior of numerous biological products, including insulin, are altered by adsorption has been indicated in previous reports (1–6). The present paper presents additional and more detailed facts concerning this behavior of insulin. When aqueous solutions of insulin are emulsified with adsorbing agents such as chloroform for a sufficient length of time, the insulin is rendered insoluble at the pH (2.5) at which insulin solutions are usually prepared. After the adsorbing agent has been removed the insulin remains as a birefringent suspension which, by subcutaneous injection into rabbits and dogs, produces a prolonged insulin reaction similar to that which is produced by other insoluble insulin preparations such as protamine zinc insulin.

The altered physical properties and physiological behavior of the insulin vary with the length of the period of treatment. After 4 hours of treatment only a negligible amount is altered, but when it is treated for longer periods of time, the insulin is rendered insoluble and becomes progressively less active with the time of treatment.

REAGENTS AND METHODS

Solutions containing 10 U per cc. of crystalline zinc insulin[2] in 0.85% buffered salt solution (pH 2.6) were sealed in pyrex glass tubes with equal volumes of the purest grade of chloroform and emulsified by shaking in a motor-driven shaking device for the desired period of time. The disc of insoluble insulin which was formed upon centrifugation was freed from chloroform under reduced pressure at temperatures below 40° C., washed with the insulin solvent, and suspended in neutral physiological salt solution. These suspensions were injected subcutaneously into rabbits, dogs and mice. The dosage indicated in the table is the actual number of units injected.

The rabbits used were adult, male, short haired albinos, weighing approximately 2 kg. each. They were well conditioned before use and kept at nearly constant weight. Food was withheld from all animals 18 hours before an experiment. No animal was used more than twice during the same week. Groups of 4 or 5 rabbits were used in each experiment, all of the animals being subjected to the same treatment.

Blood samples were taken from the ear of the dogs and rabbits and the amount of reducing substance was determined by the micro method of Miller and Van Slyke (7). When glucose was administered, sterile 10% solutions were injected intravenously. Determinations of pH were made with a glass electrode.

Control experiments, using untreated insulin, were made on all animals before

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[2] This material was made available through the generosity of Eli Lilly & Co., Indianapolis, Ind.

treated insulin was administered (group 1, table 1). Several hundred individual experiments were performed. Because of the large number of data resulting, only the averages for the groups of experiments are given.

TABLE 1. COMPARATIVE REACTION OF RABBITS TO NORMAL INSULIN AND TO DENATURED INSULIN

Group	No. of animals in group	Type of insulin	Blood sugar, in milligrams per cent					
			Normal	1 hr.	2 hr.	3 hr.	4 hr.	5 hr.
1	40	Crystalline	100	66	63	79	98	103
2	6	Treated 1 hr.	98	67	60	73	93	99
3	10	Treated 4 hr.	103	62	61	77	94	101
4	9	Treated 8 hr.	106	50	38	44	46	56
5	17	Treated 16 hr.	103	63	60	52	56	57
6	9	Treated 24 hr.	106	96	67	63	62	59
7	14	Treated 40 hr.	101	89	62	60	62	69
84	1	Treated 16 hr.	110	58	48	44	50	52
84	1	Treated 24 hr.	102	82	62	60	56	54
84	1	Treated 40 hr.	102	92	56	54	56	76

Animals of groups 1–3 received 1U of insulin; those of groups 4–7 received the residue of 20 U. of treated insulin. Group numbered 84 shows the response of an individual animal to the residue of insulin treated for different periods of time.

TABLE 2. RELATIVE RESPONSE OF NORMAL DOGS TO UNTREATED AND TO TREATED CRYSTALLINE ZINC INSULIN

Dog	Type of insulin	Insulin, Units	Blood sugar milligrams per cent					
			Normal	1 hr.	2 hr.	3 hr.	4 hr.	5 hr.
3	Untreated	2.5	96	54	50	56	58	66
3	Untreated	2.5	88	54	50	46	68	90
4	Untreated	4	98	52	48	84	84	90
4	Untreated	4	80	62	54	68	62	82
3	Treated 8 hr.[1]	25	100	78	82	50	54	50[2]
4	Treated 8 hr.[1]	40	94	74	60	50	52	44[2]
3	Treated 16 hr.[1]	25	96	86	52	46	46	56[2]
4	Treated 16 hr.[1]	40	82	92	78	52	54	60[2]
3	Treated 24 hr.[1]	50	90	80	94	88	88	86
4	Treated 24 hr.[1]	80	88	86	86	84	86	86
3	Treated 24 hr.[3]	80	92	92	102	96	88	86
3	Treated 24 hr.[4]	80	90	90	90	96	94	90
3	Treated 24 hr.[5]	80	102	92	78	76	84	86

[1] These animals received the residue of the given number of units of treated insulin.
[2] Blood sugar gradually returned to a normal level after about 12 hours.
[3] Intracutaneous.
[4] Subcutaneous.
[5] Intramuscular.

EXPERIMENTAL

The average of about 300 blood sugar determinations which were made on the normal blood of rabbits was 103 mg.%. Relatively small deviations from this average were observed among the averages of smaller groups of data and occasional larger deviations occurred among individual animals.

The data in table 1 indicate no marked alteration in the physiological behavior of crystalline insulin by treatment with chloroform for 1 and 4 hours. Treatment by adsorption for 4 hours renders a very small amount of the insulin insoluble. If at this stage the emulsion is broken up by evaporation of the chloroform under reduced

pressure and the same sample is again treated for a second period of 4 hours, an additional small amount is rendered insoluble. Continuous treatment for 8 hours, however, renders practically all of the insulin insoluble.

The data of groups 4 to 7 show the effects of treatment with chloroform for 8, 16, 24 and 40 hours on the physiological activity of crystalline zinc insulin when administered to rabbits. Insulin treated for 16 hours or longer is less effective than that treated for only 8 hours. The effect of treatment of insulin with chloroform for 8, 16, 24 and 40 hours is most evident during the first hour after its administration. This is particularly well illustrated by the data of groups 84.

The response of individual animals to treated insulin was somewhat variable since the dose administered was a border-line one. The blood sugar did not reach the convulsive level in some animals, and in others convulsions occurred at approximately 20 minute intervals for 13 hours; in still others the periods of convulsion were less frequent and less prolonged. Glucose administered intravenously disappeared rapidly and the blood sugar, which had been brought back to a normal level, again reached the convulsive level in 20 to 40 minutes. One animal into which glucose was injected at the onset of each convulsion underwent 10 such cycles before a stable, normal level was established.

The figures in table 1 show that rabbits responded to insulin treated for 24 hours, although somewhat more slowly than to that treated for 8 to 16 hours. Normal dogs, on the other hand, while responding to the action of insulin treated for 8 and 16 hours, did not respond at all to insulin treated for 24 hours, even when the dosage was doubled. The route of injection did not influence the response (table 2).

The response of mice[3] to insulin treated for 24 hours was about the same as that of rabbits. While doses of 0.015 gm. of untreated insulin were required to bring 40% of these animals to a convulsive state, the residue of 20 times this amount of treated insulin was required to produce a somewhat delayed but similar effect.

SUMMARY

Crystalline zinc insulin adsorbed by emulsions of chloroform becomes insoluble and exhibits a greatly reduced but prolonged physiological activity in mice, rabbits and dogs. Insulin treated for 24 hours and longer is reactive in rabbits and mice but not in dogs.

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[3] Mice were used in groups of 25 to 30 and were treated according to the standard technic of Trevan and Boock (8).

ISLAND HYPERPLASIA IN THE PARTIALLY DEPANCREATIZED RAT

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THE HISTOLOGIC APPEARANCE OF THE ISLANDS OF LANGERHANS in the pancreatic remnants of partially depancreatized animals is the subject of a number of reports. The most striking change which has been noted is the hydropic degeneration of the beta cells in dogs (1–4). More recently, with reference to rats rendered diabetic by subtotal pancreatectomy, Long, Katzin and Fry (5) stated: "The islet tissue of the pancreatic remnants displayed the well-known pathological changes previously described in partially depancreatized dogs. There may be found hypertrophy and all degrees of islet cell degeneration from the most minor to complete destruction and hyalinization."

It is the purpose of the present report to describe the changes in the pancreas of the partially depancreatized rat in greater detail, and to point out differences between the changes in rats and those observed in dogs.

METHODS

Twenty-three male albino rats of the Yale strain were subjected to subtotal pancreatectomy[2] at about one month of age, according to a modification (5, 6) of the method of Shapiro and Pincus (7). A small portion of the pancreas in the region of the bile duct was not removed and was disturbed as little as possible. Dextrose tolerance tests were made to determine the presence and the severity of the diabetic state. The tests were performed after an 18-hour fast with the animals under anesthesia induced by sodium pentobarbital. Three and five-tenths cc. of 10% dextrose in physiologic NaCl solution per 100 gm. of body weight were injected intraperitoneally and the blood sugar was determined by the Folin-Malmros (8) micromethod on blood secured from the tail in micropipets.

The animals were killed at intervals varying from 2 weeks to 1 year after operation and the pancreatic remnant fixed in Bouin's solution. For general study hematoxylin and eosin stains were employed, Gomori's method (9) being used to differentiate the alpha and the beta cells.

RESULTS

Changes in the islands of Langerhans. The most striking changes observed were hypertrophy of the beta cells and hyperplasia of the islands (fig. 2 and 3). The smooth contour of the islands as seen in controls (fig. 1) was broken up by the extension of masses of insular tissue between the surrounding acini, while less strikingly altered

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islets merely bulged against the adjacent tissue. An increased amount of connective tissue was present, and this sometimes gave the hyperplastic islands a lobulated appearance. There were many deposits of hemosiderin in and about the islands. The hyperplastic islands were composed almost entirely of enlarged beta cells, the alpha cells being unaltered. Hydropic degeneration was observed in a few cells in only one instance, and no hyalinization was seen.

In the first few weeks after operation and before the hyperplastic process was fully developed other changes were noted. Many island cells contained nuclei with a marked degree of vacuolization (fig. 4) and numerous mitotic figures (fig. 5) were present. During this period there was also marked hyperemia of the islands.

Acinar tissue. In the tissues from many of the animals there were large acini

TABLE 1. CORRELATION OF HISTOLOGIC AND PHYSIOLOGIC RESULTS OF PARTIAL PANCREATECTOMY IN RATS 2 WEEKS TO 1 YEAR AFTER OPERATION

Animal no.	Histologic appearance		Result of dextrose tolerance test						
	Time after operation	Island hyperplasia	Time after operation	Blood sugar (mg. per 100 cc.)					
				fasting	½ hr.	1 hr.	2 hr.	3 hr.	5 hr.
1	2 weeks	early	2 weeks	93	324	334	336	242	182
2	2 weeks	early	2 weeks	116	236	316	280	196	70
3	3 weeks	moderate	3 weeks	114	364	392	424	416	416
4	3 weeks	early	3 weeks	119	308	528	528	300	210
5	4 weeks	moderate	4 weeks	142	412	436	416	336	258
6	4 weeks	marked	4 weeks	160	392	372	—	328	192
7	6 weeks	slight	6 weeks	174	294	360	392	404	344
8	6 weeks	moderate	6 weeks	132	286	320	352	324	236
9	3 months	slight	3 months	356	420	470	464	480	440
10	3 months	none	3 months	147	300	364	472	516	492
11	5 months	marked	5 months	165	334	436	544	672	668
12	5 months	none	4 months	158	324	368	420	456	476
13	5 months	slight	4 months	—	393	448	428	404	404
14	8 months	moderate	6 months	152	304	368	448	548	496
15	8 months	moderate	3 months	133	336	444	492	520	404
16	1 year	slight	10 months	172	320	412	488	524	524
17	1 year	none	10 months	182	348	424	504	552	560
18	1 year	moderate	10 months	166	328	408	444	490	592
19	1 year	slight	6 months	204	320	376	420	434	444
20	1 year	moderate	6 months	232	404	456	508	524	488
21	1 year	none	6 months	224	416	744	524	476	532
22	1 year	slight	6 months	149	262	368	404	504	492
23	1 year	moderate	6 months	131	274	336	436	464	480

composed of large cells filled with zymogen granules, with the chromophile substance reduced to a thin band at the base of the cell. The acini were frequently concentrated in zones about islands (fig. 6). There were no dilated ducts in relation to such acinar groups. No relation between this change and the altered islands was observed, and the two types of change appeared independently in several instances. The abnormal acini were not observed before the 3rd month after operation but were present in most of the animals killed after that period.

Proliferative changes. New formation of pancreatic tissue at the edges of the pancreatic remnant was observed in many instances. In such regions there were at first numerous ductules and budding islets and acini (fig. 7). Their appearance was distinct from that of the hyperplastic islets previously described, and the latter were usually found within the substance of intact pancreatic lobules. In some cases the differentiated, newly formed pancreatic tissue showed the lobular branched structure described by Grauer (10) in the regenerating pancreas of the rabbit.

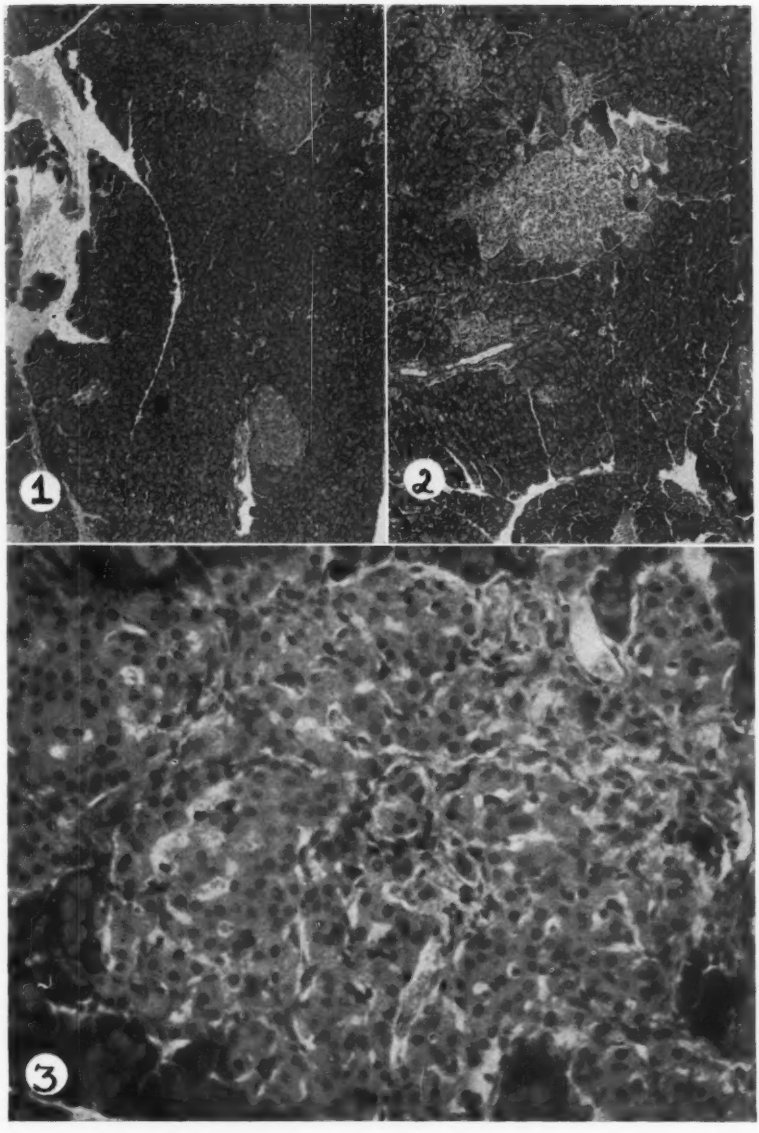

Fig. 1. Normal islets. (×50). Fig. 2. Hyperplastic islets. (×50). Fig. 3. Hyperplastic islet. (×265).

Relation of dextrose tolerance and anatomic changes. No definite relation between the degree of impairment of sugar tolerance, as measured by the dextrose tolerance test, and the degree of hyperplasia of the islands was observed. Some markedly diabetic animals had islets which appeared almost normal. It should be mentioned that impairment of sugar tolerance in rats subjected to partial pancreatectomy does

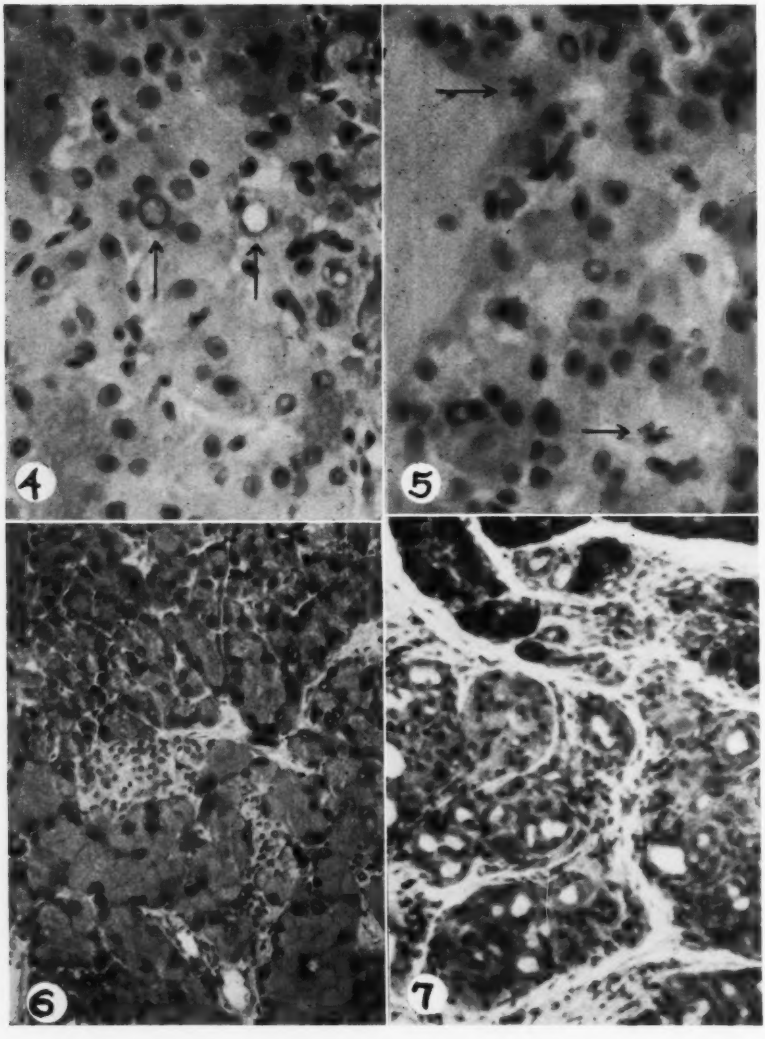

Fig. 4. EARLY CHANGES. Vacuolated nuclei. Single mitotic figure. Hyperemia. (×450). Fig. 5. EARLY CHANGES. Two mitotic figures. Hyperemia. (×550). Fig. 6. PERI-INSULAR ACINAR CHANGE. Islet normal. (×150). Fig. 7. PROLIFERATING DUCTULES WITH NEW FORMATION OF ISLAND AND ACINAR TISSUE (×160).

not develop immediately after operation but only after 3 to 6 weeks. In the table the results of the dextrose tolerance tests and the anatomic changes are listed.

DISCUSSION

Although an increase in island tissue has been reported by some workers (11–16) following the administration of insulin to normal animals, the evidence which has been presented is not convincing. Furthermore, other investigators (17, 18) found that

no hyperplasia resulted from the use of insulin. Friedman, Gomori and Caldwell (19) studied the islets of rats and guinea pigs after prolonged periods of hypoglycemia produced by protamine zinc insulin (20) and found no evidence of hyperplasia.

Hyperplastic changes in islands have also been reported following hypophysectomy (21), after administration of estrin (22), after castration (23), during hibernation (24), following injections of pituitrin (25), with vitamin B deficiency (26, 27), and following the administration of phloridzin and epinephrine (28). Several workers reported that duct ligation was followed by hypertrophy and hyperplasia of the islands (29–31). The increase in island tissue reported by Cameron (32) and Watrin and Florentin (33, 34) after the administration of thyroid substance and of thyroxin was not observed by Haban and Angyal (35).

Woerner (36), by prolonged intravenous injections of dextrose in the guinea pig, produced an extensive increase in island tissue which was especially marked in one partially depancreatized animal.

In three reports (37–39) on alterations in the islands the changes described are similar to those herein reported. The studies were all carried out on rats given anterior pituitary extract. Figure 5 in the paper by Richardson and Young (39) also shows comparable acinar changes.

The observations in the present study suggest that the dominant reaction on the part of the islands of Langerhans in the partially depancreatized rat is one of hyperplasia, with degenerative changes playing a minor role. Conversely, in the dog, although some proliferative changes take place after partial pancreatectomy, the dominant changes are those of degeneration. It must be noted, however, that in most of the work with dogs, adult animals were employed and that the response of the islands in young growing animals might be different. Homans (4) pointed out that hypertrophy of the pancreatic remnant was especially marked in young dogs.

The present observations are consistent with the suggestion of Marks and Young (40) that the pancreatic mechanisms of the rat are more sensitive than those of the dog. There are similar contrasts in the reactions of the dog and the rat to other procedures. Administration of pituitary extract causes predominantly hyperplastic changes in the rat pancreas (39), with increase in the insulin content (40, 41) while in the pancreas of the dog depression of the insulin content (42) and degenerative changes (43) predominate. Lacoste, Aubertin and Saric (44) found that the administration of insulin causes an increase in the insulin content of the dog's pancreas while such administration depresses the pancreatic insulin content of the rat (45).

SUMMARY

Hyperplasia of the islands of Langerhans involving the beta cells was observed in the pancreatic remnant of partially depancreatized rats. There was no correlation between the degree of hyperplasia and the degree of impairment of sugar tolerance. The anatomic changes observed are similar to those which have been reported by other workers following the injection of anterior pituitary extracts. The difference in the reaction of the residual islet tissue of the dog and of the rat to partial pancreatectomy is pointed out.

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THE EFFECT OF GLYCOLYSIS INHIBITORS AND OF CERTAIN SUBSTRATES ON THE METABOLISM AND MOTILITY OF HUMAN SPERMATOZOA

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SEVERAL STUDIES ON THE RELATION BETWEEN the metabolism and motility of mammalian spermatozoa (1–6) show that motility is maintained for extended periods under anaerobic conditions and that substances such as cyanide and carbon monoxide which specifically inhibit respiration do not have any effect on motility. In 1935, Iwanow (7) further showed that concentrations of mono halo acetic acids which inhibited the glycolysis of dog spermatozoa did not destroy motility, and he concluded that neither glycolysis nor respiration was an essential source of energy for motility. Lardy and Phillips (4) in a study of bull spermatozoa used monoiodoacetic acid, and confirmed Iwanow's observations. Recently, the same authors have presented evidence which indicates that, in the presence of oxygen, phospholipids may act as a source of energy for bull spermatozoa.

The rôle of substrates in the motility of mammalian spermatozoa has not received much attention though Iwanow (8) has found that the motility of sheep spermatozoa is not dependent upon the presence of glucose. Redenz (2), on the other hand, pointed out that the motility of bull and guinea pig spermatozoa ceased in glucose-free Ringer's solution and could be stimulated again by the addition of glucose. It is generally assumed (9, 10) that the high glucose content of seminal fluid acts primarily as a substrate for the spermatozoa, but this assumption, particularly in the case of human semen, has not been subjected to quantitative experimental analysis. In this report, it will be shown that human spermatozoa are dependent entirely upon utilizable carbohydrate as a source of energy for motility and that the essential metabolic pathway for the breakdown of carbohydrates is that of glycolysis, the splitting of glucose to lactic acid.

METHODS

Aerobic and anaerobic glycolysis was measured by the Warburg technic at 38° C. as described more fully in a previous paper (6). For the determination of the effect of glycolysis inhibitors, the spermatozoa were separated from the seminal fluid by diluting the semen about 5 times with Ringer-glucose solution and centrifuging for 10 minutes at 1,500 R.P.M. The packed cells were then re-suspended in Ringer's solution containing 100 mg.% glucose and 0.03 M bicarbonate before the transference of measured volumes to the manometer vessels. Monoiodoacetate and sodium fluoride were the glycolysis inhibitors used. Known concentrations of these substrates were placed in the side-arms of the manometer vessels so that they could be added to the spermatozoa suspensions in the main vessels at any chosen time during the experi-

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ment. Normal glycolysis was measured for one hour and in most experiments, the iodoacetate or the fluoride was added at this time. Since motility will be discussed in detail, it is necessary to state how it is determined qualitatively and quantitatively. Quality is expressed arbitrarily as 4, 3, 2, 1 and 0. Four represents maximal motility and 1 a movement which may be expressed best as a sluggish undulation around a fixed point. The determination of the quality of motility is a matter of experience, and a trained observer can do this rapidly. Quantitatively, motility is expressed as the % of motile cells, and this is determined in the following manner: a binocular microscope is used, one eye-piece of which is prepared so that only one-quarter of the field is illuminated. The observer examines the cells in the full high-power field to determine

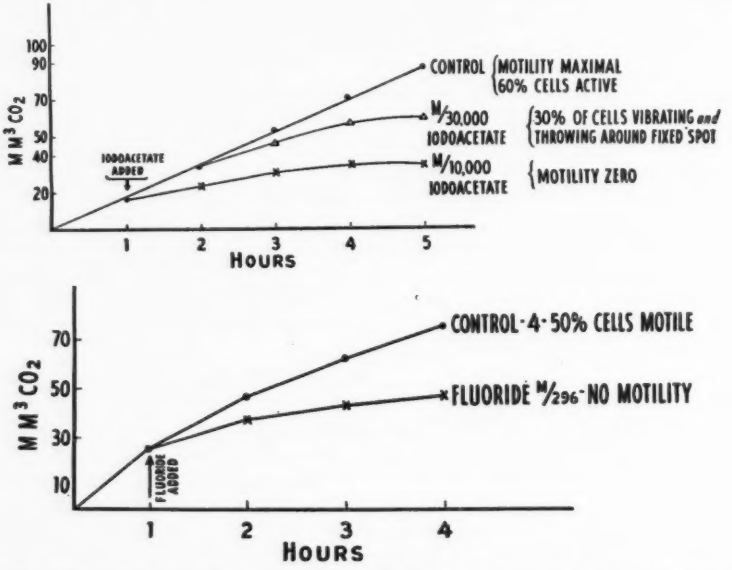

Fig. 1. Effect of sodium monoiodoacetate upon anaerobic glycolysis and motility of human spermatozoa. Fig. 2. Effect of sodium fluoride upon aerobic glycolysis and motility of human spermatozoa.

the quality of motility, and then, transferring his eye to the quarter-field eye-piece, counts the number of cells crossing the field under their own power in 10 seconds. This number divided by the total number of cells in the field gives the percentage of motile cells. The final figure is only an approximation but we have found, after several hundred examinations, that it is reliable within $\pm 10\%$. Motility was determined at the beginning and conclusion of each experiment.

To determine the effect of various substrates and of the absence of substrate upon the metabolism and motility, it was necessary to modify the above technic. The semen was diluted 5 times with glucose-free Ringer's solution, centrifuged, and the packed cells re-suspended in glucose-free Ringer's solution. Since the cells were washed only once in order to avoid undue injury it was inevitable that a small amount of glucose would remain in contact with the packed cells but this amount was negligible and in most experiments was constant. For all practical purposes, we have assumed that, under these conditions, the cells transferred from the semen to the glucose-free Ringer's solution carried with them only a very small amount of glucose.

In these experiments, glucose was added to the side-arms of the manometers in such concentration that, when added to the spermatozoa suspensions in the main vessels, the final concentration would be 100 mg.%. In certain experiments, other carbohydrates such as fructose, mannose, galactose, and maltose, were substituted for glucose. Aerobic and anaerobic glycolysis was measured over a period of several hours, and the sugars added at various times during the course of the experiment. A control vessel contained a spermatozoa suspension to which glucose had been added at the beginning of the experiment.

Effect of Monoiodoacetate and Fluoride

Figure 1 demonstrates the effect of 2 concentrations of iodoacetate upon anaerobic glycolysis and motility. M/10,000 iodoacetate rapidly and completely inhibited gly-

TABLE 1. EFFECT OF VARIOUS CONCENTRATIONS OF MONOIODOACETATE UPON GLYCOLYSIS AND MOTILITY OF HUMAN SPERMATOZOA

Expt	Total sperm count in millions	M/6000	M/10,000	M/30,000	M/40,000	M/50,000	M/65,000
1	63	Motility and glycolysis destroyed	Motility and glycolysis destroyed	Motility markedly affected and glycolysis destroyed		Motility markedly affected and glycolysis destroyed	
2	108	Motility and glycolysis destroyed	Motility and glycolysis destroyed	Motility and glycolysis destroyed	Motility holds up well. Little effect on glycolysis		
3	106			Motility and glycolysis destroyed	Motility and glycolysis affected markedly	Motility and glycolysis affected markedly	Motility and glycolysis affected

colysis and destroyed motility. On the other hand, M/30,000 iodoacetate took somewhat longer (nearly 3 hours) to produce complete inhibition of glycolysis and when, in this case, motility was determined at the end of the experiment, many of the cells, at first glance, were active. However, as shown in figure 1, when the motility of the cells in M/30,000 iodoacetate was compared with the control motility, it was evident that in the presence of iodoacetate, about 50% of the formerly active cells were completely inert and the remainder were severely affected and had lost all forward motion. In other words, while it can be said in this case that glycolysis had ceased and some motility remained, it was obvious that the source of energy has been so affected that the spermatozoa could no longer function in their normal manner.

Effects similar to these have been obtained in other experiments with higher dilutions of iodoacetate and these are outlined in table 1. In every case, the failure of glycolysis in the presence of iodoacetate was followed by a more or less complete failure of motility. Where no effect on glycolysis was evident, no marked effect on motility was apparent. The effect of iodoacetate upon aerobic glycolysis was similar to that of anaerobic in so far as the time and extent of inhibition was concerned and figure 1 may be considered representative of both. However, in certain experiments, we ob-

served that the effect of iodoacetate upon the motility may not have been as destructive when the cells were exposed to oxygen. The difference in the two effects was apparent but not of great magnitude.

The effect of fluoride upon motility and glycolysis was similar to that of iodoacetate, though much higher concentrations (relative to iodoacetate) of this substance must be used. Figure 2 shows the effect of M/296 fluoride upon aerobic glycolysis. It will be seen that the inhibition was rapid and complete and was followed by failure of motility. With fluoride, identical effects were obtained under anaerobic conditions. Higher dilutions of fluoride had little effect on glycolysis and in such cases, motility was unimpaired.

Effect of Deprivation of Substrate upon Metabolism and Motility

When human spermatozoa were suspended in glucose-free Ringer's solution in Warburg vessels at 38° C., the initial glycolysis was low and ceased completely within

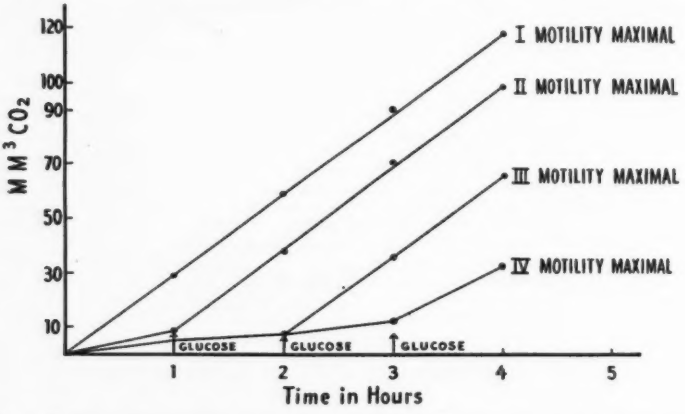

Fig. 3. Effect of deprivation of glucose and of addition of glucose at various intervals upon anaerobic glycolysis and motility of human spermatozoa. Gas phase = 95% N_2 − 5% CO_2. Motility determined at end of experiment.

3 hours. This failure of glycolysis was coincident with failure of motility, but the normal level of glycolysis and rate of motility could be restored if a utilizable sugar was added to the medium within a reasonable time after deprivation of substrate. This reversal of loss of glycolysis and motility was dependent upon the nature of the gas phase to which the cells had been exposed during the period of deprivation. These points are demonstrated in figures 3 and 4. In nitrogen (fig. 3, curve I), lactic acid production in the presence of glucose remained linear for several hours. When glucose was withdrawn, glycolysis during the first hour of withdrawal was only about 20% of the control, but if glucose was added at the end of the first hour, the control level of glycolysis was attained almost immediately. When motility was determined at the end of the experiment, it was found to be normal. The addition of glucose at the end of the second hour in nitrogen produced identical results (fig. 3, curve III) but after the third hour of glucose deprivation, the recovery of glycolysis was not complete though the motility at the end of the experiment was found to be maximal. Figure 4 demonstrates the same experiment performed in 95% O_2–5% CO_2. The effects of withdrawal of glucose under these conditions however, were quite differ-

ent.[2] Aerobic glycolysis in the presence of sugar tended to fall off with the passage of time. When glucose was withdrawn, lactic acid production was at about the same level as that of the cells in nitrogen and when glucose was added at the end of one hour, the recovery of glycolysis was similar to that in nitrogen. But in spite of the complete recovery of glycolysis, the spermatozoa had already been affected by the brief absence of glucose as was evidenced at the end of the experiment by a depression of motility and a drop in the percentage of motile cells. Lastly, curves III and IV of figure 4 demonstrate that the addition of glucose at the second and third hours respectively to the spermatozoa in oxygen initiated only a faint increase in the rate of glycolysis and did not promote any marked recovery in motility.

The inhibitory effect of oxygen upon motility and glycolysis under these condi-

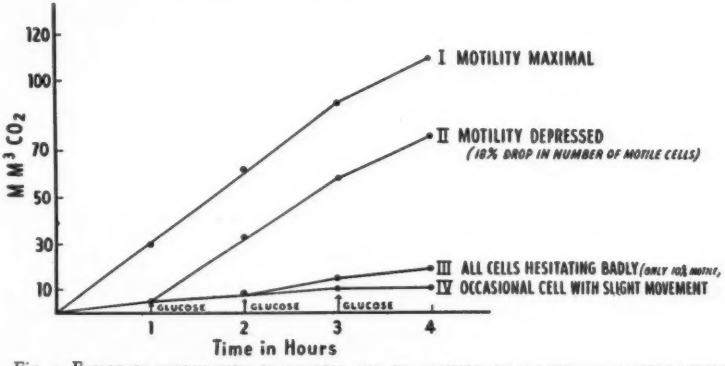

Fig. 4. Effect of deprivation of glucose and of addition of glucose at various intervals upon aerobic glycolysis and motility of human spermatozoa. Gas phase $=95\%\ O_2-5\%\ CO_2$. Motility determined at end of experiment.

tions is striking when compared to the anaerobic behavior of the spermatozoa and it complements the observations of MacLeod (6) and of Ross et al. (11) on the spontaneous loss of motility which often appears at high oxygen tensions.

Effect of Various Substrates upon Recovery of Motility

These results indicated a method whereby the effect of substrates other than glucose could be determined because it followed that if energy for motility was obtained from protein or fat metabolism then the addition of suitable substrates should initiate motility when the latter had been lost in a substrate-free medium. The results of such experiments can be stated briefly. Only utilizable carbohydrates were of any value in promoting recovery of motility; neither amino- nor fatty acids had the slightest effect. Nor could we confirm for human spermatozoa the observations made by Lardy and Phillips (4) on bull spermatozoa that phospholipids were oxidized and had a beneficial effect on motility.

Of the monosaccharides, fructose and mannose were found to be just as effective as glucose in maintaining maximal motility after the spermatozoa had been deprived of substrate. Galactose was ineffective. Among the disaccharides, maltose acted as a complete substitute for glucose, but neither sucrose nor lactose was effective. When pure glycogen was substituted for glucose (fig. 5) the initial glycolytic rate was the

[2] The data represented in figures 3 and 4 were obtained from the same suspension of spermatozoa,

same as if no added substrate were present, but the rate increased progressively until between the 4th and 5th hours, almost as much lactic acid being produced from glycogen as from glucose. Motility was retained maximally in the presence of glycogen.

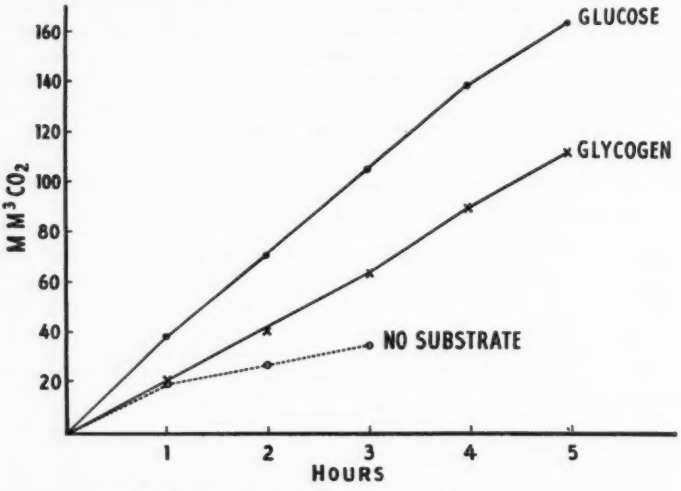

Fig. 5. Effect of a glycogen substrate upon anaerobic glycolysis.

Duration of Motility at Body Temperature in a Glucose-Free Medium

Since the motility of the spermatozoa is dependent upon an environment containing utilizable sugar, it was a relatively simple matter to determine how long motility could survive at body temperature in a sugar-free environment and at what stage recovery of motility could be induced by the addition of glucose. Table 2 presents the average of observations made in 20 experiments in which the spermatozoa of several individuals were combined in glucose-free Ringer's solution at 37.5° C. and examined for failure of motility at various intervals up to 6 hours. The observations were made in 95% oxygen and in nitrogen. Figures are given for complete loss of motility, the time at which complete recovery could be induced by the addition of glucose, and the time at which no recovery of motility could be induced by glucose.[3]

Table 2. Effect of oxygen and of anaerobiosis on the motility of human spermatozoa deprived of substrate at 38° C. Recovery upon addition of substrate

Gas Phase	Mean time for loss of motility in minutes	Mean time for complete recovery of motility upon addition of glucose	Mean time at which no recovery of motility is noted
95% Nitrogen	252	268	320
95% Oxygen	205	186	233

[3] The recovery phenomenon can be easily observed by placing a drop of the suspension of inert cells on a slide and adding a drop of 1% glucose solution. If the sperm have been kept under low oxygen tension during the period of glucose deprivation and have not been inert for longer than 30 minutes, recovery of motility is attained within a few minutes. In many experiments, spontaneous recovery of motility may be seen when the spermatozoa are placed between slide and cover-glass and before glucose is added. This

In several experiments, motility in the sugar-free medium failed completely within 2 hours. In others, a feeble motility continued up to six hours. In every case, motility was obviously affected shortly after the cells were deprived of glucose. Under anaerobic conditions, the spermatozoa withstood lack of substrate for periods ranging from 4 to 7 hours and still retained the ability to recover active motility in glucose. But their ability to recover in 95% oxygen was lost within from 2 to 3 hours. Glycogen could be substituted for glucose in inducing recovery of motility but, as in the glycolysis experiments, a decided 'lag period' was evident and the recovery was not as general as with glucose.

DISCUSSION

From the metabolic viewpoint, the effect of iodoacetate and of fluoride upon the glycolysis and motility of the spermatozoa offers an interesting comparison with the effects of the same substances on the lactic acid production of skeletal muscle. The work of Embden and Haymann (14), Davenport and Cotonio (15), and Embden and Deuticke (16) on skeletal muscle has demonstrated that in the presence of fluoride, hexose-di-phosphate can be formed and broken via triosephosphate to phosphoglyceric acid but that there is no further conversion to pyruvic acid and free phosphate. Adler, von Euler, and Gunther (17) have shown that iodoacetate acts upon the triosephosphate dehydrogenase and prevents the breakdown of hexose-di-phosphate. Though there is no conclusive evidence as yet that glycolysis in human spermatozoa follows the same pathway as that in skeletal muscle, the fact that lactic acid formation in the spermatozoa is inhibited by substances producing similar effects in muscle is strong presumptive evidence that a similar metabolic pathway exists in both tissues.

From the evidence presented here it is apparent that human spermatozoa are dependent upon utilizable carbohydrate for motility and that they cannot long be deprived of it. It is also apparent that any factor interfering with the breakdown of glucose to lactic acid in the spermatozoa destroys their motility. This evidence is not in harmony with the work which demonstrates that the motility of dog (7) and of bull (4) spermatozoa is maintained in the presence of iodoacetate and in the absence of glycolysis. The different behavior of human and bull spermatozoa in the presence of iodoacetate would not seem to rest upon a qualitative difference in metabolic function since Lardy and Phillips[4] agree that, as in human spermatozoa, the aerobic and anaerobic glycolysis of bull spermatozoa are quantitatively identical and that the oxygen consumption is very low. It is more probable that the difference may rest upon a definition of active motility or upon the energy relationship which determines how much glycolysis is necessary for active motility. For example, while it is true that in the case of human spermatozoa, some cells may show a feeble motile activity in the presence of iodoacetate and fluoride after glycolysis has ceased, it does not follow that the spermatozoa are deriving energy from a source other than glycolysis. The amount of energy in terms of lactic acid formation for such feeble activity is quite probably within the limits of error of the manometric and chemical determinations of lactic acid. Then, too, it is possible that some energy can be derived for motility from a

type of recovery seldom takes place if the cells have been exposed to oxygen for any length of time. Recovery is invariably found only when the cells have been kept under anaerobic conditions. This spontaneous recovery lasts only a few minutes and is in no way comparable to the recovery of motility which takes place on addition of glucose.

We have no explanation for this phenomenon of spontaneous recovery of motility between slide and cover-glass, but it may be related to the sphering of erythrocytes under the same conditions described by Ponder (12). Furchgott (13) has since demonstrated that this sphering effect is due, in great part, to a remarkable shift of pH to the alkaline side which takes place in solutions placed between slide and cover-glass.

[4] Personal communication.

breakdown of carbohydrate to a point short of lactic acid. Lastly, it has been shown (18) that human spermatozoa contain appreciable amounts of creatinphosphate and that this substance is broken down and resynthesized by these cells. It is possible, therefore, that, as in skeletal muscle, the hydrolysis of creatinphosphate may furnish energy for a time after glycolysis has ceased. This phase of the metabolism is now under investigation in this laboratory.

The necessity of utilizable carbohydrate for the continuance of active motility makes it clear, at least for human spermatozoa, that the energy for motility is derived exclusively from some form of carbohydrate metabolism. This dependence of the spermatozoa upon sugar is of importance in considering the life of the spermatozoa in the male genital tract and their existence after leaving the seminal fluid in the female genital tract. The excellent work of Young and his co-workers (19–22) makes it clear that, in the mammal, the post-testicular history of spermatozoa is such that they may remain in the epididymis and ductus deferens for periods as long as 30 days and still retain their capacity for motility and fertilization. It is not to be expected that they are actively motile during this time and several theories (23, 24) have been advanced to explain why motility is suppressed prior to the mixing of the spermatozoa with the seminal fluid at the time of ejaculation. None of those theories has considered the possibility that prior to ejaculation, the spermatozoa may not have access to sufficient substrate and that they do not come in contact with large amounts of glucose until they reach the secretions of the seminal vesicles. Huggins (25) has shown that spermatocoele fluid contains virtually no carbohydrate.[5] While we have no positive evidence that a low glucose level in the epididymis and ductus deferens may serve to depress motility and to keep the spermatozoa at a low energy level during their existence prior to ejaculation, such a speculation is worthy of further consideration. It is certain that the mere addition of glucose is sufficient to stimulate inert spermatozoa to intense activity within a few seconds and it is not necessary to postulate any substance in the seminal fluid more complex than sugar as the initiator of the intense spermatozoa motility found in that environment.

Lastly, in regard to *in vivo* conditions in the female genital tract, the question of available substrate for the spermatozoa is probably of prime importance. Though little is known of the fate of the human spermatozoa after deposition of the semen in the vagina, Carey (26) has reported the presence of live spermatozoa in the cervix 36 to 48 hours after coitus and Moench (27) has found the time for survival of spermatozoa in the uterus and tubes to be from 36 to 48 hours. In view of the evidence presented in this paper on the survival of motility after the spermatozoa have been deprived of sugar, it is clear that the amount of substrate (glucose) carried by the spermatozoa on leaving the seminal fluid is very limited and that they must have access to substrate almost immediately and have that access continuously until they fertilize the ovum. So far as can be determined, this question has been ignored by workers in the field though much speculation has been made (28) as to the significance of the high glycogen content of the endometrial epithelium at certain stages of the menstrual cycle. Since the spermatozoa can use glycogen and since they must have access to substrate for motility, it is possible that an abundance of uterine glycogen plays a rôle in nutrition of the spermatozoa in their passage to the tubes.

SUMMARY

Monoiodoacetate and fluoride inhibit the aerobic and anaerobic glycolysis of human spermatozoa and have a correspondingly depressing effect upon the motility

[5] We have confirmed this observation and can add that the spermatozoa found in spermatocoele fluid show no motile activity.

of these cells. The motility is dependent upon the energy derived from the breakdown of sugar to lactic acid. No other substrates are effective.

Time relationships are given for the loss of motility which takes place at 38° C. when the spermatozoa are deprived of utilizable carbohydrate and for the recovery of motility which follows the addition of sugar to the extra-cellular environment.

The possible application of these time relationships to the behavior of human spermatozoa *in vivo* in the male and female genital tracts is discussed.

The technical assistance of Miss Eleanor Sayer throughout the course of this work, particularly in the determination of motility, has been of the greatest value.

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GONAD RESPONSE OF MALE RATS TO EXPERIMENTAL HYPERADRENALISM

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ADRENALIN HAS BEEN SHOWN TO SUPPRESS the reproductive activity of the English sparrow (1), presumably by antagonizing the pituitary gonadotropins. Gonads, accessory structures and secondary sex characteristics appear to share in the regression produced. The seasonal reproductive activity in the sparrow has been correlated with the amount of circulating adrenalin. The purpose of this study was to determine if adrenalin would suppress the reproductive activity in animals which continually breed, and, if so, to find what part adrenalin normally plays in such animals. Robson (2) reported that adrenalin interfered with the estrous cycle of the mouse. The present work extends the study of the inhibition of the gonads by adrenalin to the white rat.

EXPERIMENTAL PROCEDURE

Daily injections of 1 to 4 minims of adrenalin chloride (1:1,000)[1] were given subcutaneously to 53 male rats from 15 different litters. Seventeen of these animals served as controls for the various litters. One control for each group was killed at the outset of the experiment and a second at the conclusion. After 14 days of treatment the experimental animals were periodically killed at intervals of 7 to 10 days depending upon the response of the animal to treatment. The animals were maintained on a standard rat diet[2] during the course of the experiment.

At autopsy the reproductive organs were fixed in Bouin's fluid. The tissues were dissected free, cleaned, and weighed after they had been transferred to 70% alcohol. Sections of the right testis and epididymis were prepared for microscopic study. In some instances the seminal vesicles, prostates, pituitaries and adrenals were also prepared for study.

RESULTS

The weights of the reproductive organs are given in table 1. The accessory reproductive organs weighed less in the treated than in the untreated control animals. No sperm were seen in the epididymis of the 8-week-old rats and there were but a few in those of the 9-week-old experimental and control animals.

In table 1 it is apparent that the accessory reproductive tissues of the experimental animals in *groups III* to *VII* weighed less than those of the controls. The animals in groups which received the larger amounts of adrenalin for longer periods of time had relatively heavier weights recorded for the reproductive organs than the experimental animals receiving one-half the dose of adrenalin for shorter periods. The regression of the testis was marked in the experimental animals of *group VI*.

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[1] Parke, Davis and Company, Detroit, Mich.

[2] Diet prepared by Maritime Milling Company, Buffalo, N. Y.

The individual differences between control and experimental animals were of the same order as the group averages indicate. Seven experimental animals had testis weights below 0.4 gm.; 11 were between 0.4 and 1.0 gm.; 10 testis weights were slightly larger than the control weight, while 4 were considerably larger. The rats of *group VI* all of which had testis weights below 0.4 gm. were of particular interest, since these animals were injected during the months of July and August. Two other animals with very low testicular weights were injected during April and October.

TABLE 1. WEIGHT AVERAGES OF REPRODUCTIVE ORGANS IN ADRENALIN-INJECTED AND CONTROL RATS. Weight loss of experimental animals expressed in percentage.

Group	Rats number	Injections No.	Injections Minims	Age limits	Weights in gm. Body	Testis	Epidid.	Prostate	Sem. Ves.
I.	3 exp.	14	1	6–8 wk.	155.9	0.719	0.063	0.125	0.0226
	2 c'tl.			8 wk.	155.0	0.782	0.092	0.291	0.0670
		exp. loss			0.58%	8%	31%	57%	36%
II.	3 exp.	21	1	6–9 wk.	197.2	1.075	0.146	0.294	0.094
	1 c'tl.			9 wk.	221.5	1.149	0.279	0.375	0.145
		exp. loss			10.4%	6.4%	47.6%	22%	35%
III.	9 exp.	14–21	2	2–3 mo.	191.0	0.868	0.161	0.193	0.079
	2 c'tl.			2 & 3 mo.	246.0	1.217	0.293	0.755	0.386
		exp. loss			22%	28%	41%	75%	80%
IV.	8 exp.	14–21	2	3–4 mo.	244.4	1.021	0.292	0.388	0.222
	2 c'tl.			4 mo.	315.0	1.187	0.453	1.012	0.468
		exp. loss			22.5%	14%	35.5%	61.6%	52.5%
V.	13 exp.	28–42	2	3–4 mo.	220.0	1.119	0.288	0.412	0.239
	3 c'tl.			4 mo.	316.0	1.269	0.437	0.955	0.427
		exp. loss			30%	11.7%	34%	56.8%	44%
VI.	5 exp. summer rats	28–42	2	2–3 mo.	217.0	0.350	0.130	0.241	0.171
	1 c'tl.			3 mo.	274.0	1.290	0.405	0.840	0.383
		exp. loss			21%	72.8%	68%	65%	55%
VII.	4 exp.	60	2	4–6 mo.	345.0	1.119	0.326	0.576	0.171
	3 c'tl.			6 mo.	320.0	1.216	0.396	0.954	0.533
		exp. loss			7.2%	7.9%	17.6%	39.6%	67.8%
VIII.	8 exp.	30–60	4	4–6 mo.	276.0	1.096	0.337	0.728	0.295
	3 c'tl.	(same as group 7)			320.0	1.216	0.396	0.954	0.533
		exp. loss			13.7%	9.8%	14.8%	39.6%	42%

In the 7 animals with low testis weights there was severe cellular damage in the testis. Weight loss was more constant in the accessory reproductive organs than it was in the testes. In no instance did the weights of the seminal vesicles in the experimental animals exceed that in the controls. The weights of the prostates in the experimental groups exceeded the weight of the controls in 5 instances; the weights of the epididymis were greater in the experimental group in only 4 instances.

Histological study revealed a correlation between weight loss and microscopic structure. In the 7 testes of extremely low weight there was a complete suppression of spermatogenic activity (fig. 1). Pronounced regression occurred in 4 other specimens, although many tubules were apparently still active, since they contained sperm. In 10 animals there were regressive changes in many testis tubules. In 9 other

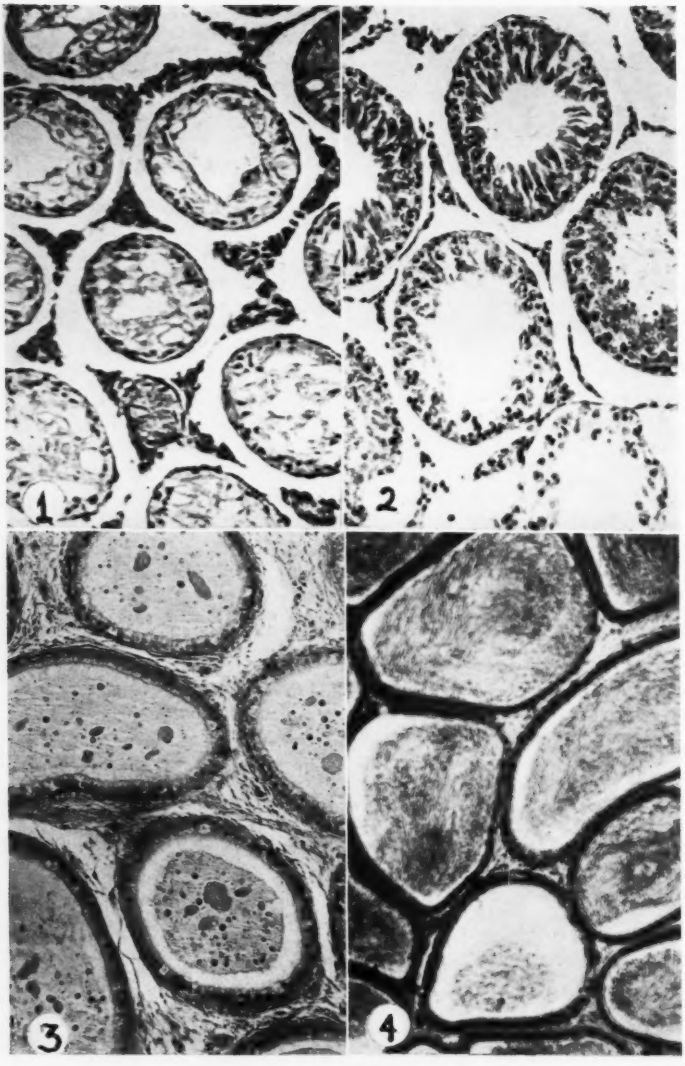

Fig. 1. TESTIS EXPERIMENTAL, RAT. Complete spermatogenic disintegration; summer experiment. ×50. Fig. 2. TESTIS EXPERIMENTAL, RAT. Winter experiment; 2 tubules regressing. ×50. Fig. 3. EPIDIDYMIS OF SAME ANIMAL AS IN FIG. 1. Absence of sperm and phagocytic cells. ×50. Fig. 4. EPIDIDYMIS OF SAME ANIMAL AS IN FIG. 2. Some evidence of dilution, tissue normal in other respects. ×50.

animals there were tubules in which there was diminution of spermatogenesis. In the remainder of the experimental animals there was no visible testis damage. Where there was complete regression of the testis there were no sperm in the epididymis. Where the regression was incomplete, there was a marked diminution in the number of sperm in the epididymis. Frequently, where sperm production was not evident in

the testis, a few sperm were found in the epididymis. Phagocytosis was generally observed in the sections of the epididymis of experimental animals (fig. 3).

In the prostate and seminal vesicles, measurements showed the epithelium to be low. This appeared to bear some relationship to the degree of weight loss. However, in cases of severe testis damage many tubules of the prostatic epithelium continued to exhibit the cytoplasmic 'moons' characteristic of the organ. Cowper's glands were not studied histologically in all cases, but there were atrophic changes apparent in the sections which were studied.

DISCUSSION

From the data obtained in this study, it appears that damage to the testis of rats following administration of adrenalin varied considerably in degree. The seminal vesicles, prostate, epididymis and vas deferens were atrophied after such treatment. In the female rat, diestrous periods of 5, 7, 10, 14, 21 and 29 days have been observed after treatment with adrenalin (3). The same type of finding has been reported for mice (2).

In male rats under conditions of experimental hyperthyroidism (4), there was no testicular damage, although there was some atrophy of the accessory reproductive organs. This result may have been produced by an increase in the production of adrenalin in the experimental animals, rather than by the direct action of the thyroid hormone.

In the sparrow the antigonadal action of adrenalin has been considered as acting primarily against the pituitary gonadotropic hormones (1), and thus secondarily against the sex hormones. Robson found the same in mice (2). However there has been no histologic demonstration of a pituitary involvement under such conditions by the writer.

The cessation of spermatogenesis in rats receiving adrenalin during the summer months is interesting, since adrenalin is known to have a calorigenic action (5). The mammalian testis undergoes a degeneration when external heat is applied which is comparable to the degeneration observed in cryptorchidism (6). In the high environmental temperatures of the summer months, an increase in the general body heat production induced by adrenalin may have played a part in the depression of spermatogenesis.

The atrophy of the testis produced by adrenalin in this study differed from cryptorchidism, since there was also an atrophy of the accessory organs of reproduction in the animals which received adrenalin.

SUMMARY

Experimental hyperadrenalism in the male rat brought about a regression of the accessory organs of reproduction, and in some instances completely suppressed spermatogenesis.

There is a difference in the response of the testes of the rat and of the sparrow to experimental hyperadrenalism.

The author wishes to express his gratitude to Dr. Oliver Kamm of Parke, Davis & Co., Detroit, Mich. for the adrenalin chloride used in these experiments.

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THE EFFECT OF ENDOCRINES ON THE FORMED ELEMENTS OF THE BLOOD[1,2]

I. The Effects of Hypophysectomy, Thyroidectomy and Adrenalectomy on the Blood of the Adult Female Rat

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THE ENDOCRINE GLANDS may exert an influence on the formed elements of the blood. In the classical paper of Aschner (1) a decreased erythrocyte count and a decreased hemoglobin content in the blood of a hypophysectomized dog was reported. Since then several clinical (2–10) and experimental reports have been published supporting the theory that the hypophysis is concerned with the process of erythropoiesis.

Houssay, Royer and Orias (11) observed an anemia and a decrease in hemoglobin after hypophysectomy in the rabbit. Stewart, Greep and Meyer (12) found that the hypophysectomized rat would not respond to a decreased oxygen tension with the typical bone marrow hyperplasia, reticulocytosis and an increased red count which is found in normal rats under similar conditions. Meyer, Stewart, Thewlis and Rusch (13) concluded that hypophysectomy in the rat produced a reticulocytopenia and an anemia. Vollmer, Gordon, Levenstein and Charipper (14) confirmed the above results on the rat, finding a decrease in the red count, hemoglobin and reticulocytes and a bone marrow hypoplasia after removal of the hypophysis.

Querido and Overbeck, in a series of papers (15–18), at first found no anemia after hypophysectomy but did find a reticulocytopenia which could be prevented by injections of a crude extract of the anterior pituitary gland. Removal of the thyroid, adrenals, or spleen failed to produce this same decrease in reticulocytes. Again assuming no anemia (18) these authors found that both hypophysectomized and normal rats responded to bleedings and to phenylhydrazine by a reticulocytosis, and thus concluded that the pitiutary was concerned with blood destruction rather than with blood formation.

Flaks, Himmel, and Zlotnik (19, 20) obtained in the rat an increase in hemoglobin, an increase in the red count, and a reticulocytosis with extracts of anterior pituitary from which the growth, gonadotropic, and thyrotropic hormones had been removed by boiling at 100° for 5 minutes. These authors claim their extracts are effective when administered orally. They conclude that the pituitary is concerned with blood production by directly regulating the bone marrow.

On the other hand, Meyer, Thewlis, and Rusch (21) have reported that a reticulocytosis can be produced in the rat by various hormones and also by inert materials. They conclude therefore "that the hypophysis does effect hemopoiesis, but that the effect appears to be associated with general metabolic changes and probably is not dependent upon a specific hormone acting upon the bone marrow."

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[2] Aided by a grant from the Rockefeller Foundation, administered by Dr. P. E. Smith.

[3] Columbia University Research Fellow in the School of Medicine. Now located at Boston University Medical School, 80 E. Concord St., Boston, Mass.

Certain organs are known to atrophy after hypophysectomy, but it is not known whether the pituitary gland exerts its influence on erythropoiesis directly or secondarily through the atrophy of the thyroid or the adrenal glands.

The present report is the result of an attempt to analyze further the various endocrine factors involved in the formation of blood cells of the rat by a comparison of the effects obtained in the cellular blood picture by hypophysectomy, adrenalectomy (+1% NaCl), and thyroidectomy.

It is generally agreed that adrenalectomy results in a rise rather than a decrease in the red count (23–25). However, the hemoconcentration resulting from adrenal cortex removal is quite possibly the cause for this rise. The adrenals may have an influence upon hemopoiesis as is indicated by the frequent occurrence of anemia in patients with Addison's disease (22).

In 1904 Kishi (26) reported an anemia after complete thyroidectomy in the cat, dog, and the ape. Kunde (27–29) also reported an anemia after thyroidectomy in rabbits characterized by a color index greater than 1. Several papers report a retarded recovery from experimental anemia in the absence of the thyroid gland (30–32). In a review of the literature Hoskins and Sleeper (33) listed several clinical reports which are uniform in finding an anemia in hypothyroidism. Askanazy (34) reported that thyroidectomized animals did not show the normal polycythemic reaction at pressures lower than atmospheric while Meyer, et al. (21) obtained a normal reticulocyte response under similar conditions.

METHODS

Adult female rats of 3 to 4 months of age and weighing 100 to 150 gm. were employed in this study. Hypophysectomy was performed by the parapharyngeal approach. The completeness of the removal was determined by examination of serial sections of the pituitary capsule stained with Masson trichome. Adrenalectomy was done by the dorsal approach and, in addition to the glands, all fat in the area was thoroughly extirpated. The completeness of the thyroidectomy and the adrenalectomy was determined at autopsy by a careful examination of the respective areas under the binocular microscope. Several thyroid remnants and one adrenal remnant were found in this manner. These animals are not included in these data. Other animals were subjected to 'control operations,' all details of operative technic being repeated except the actual removal of the glands. Ether anesthesia was used for the surgery. The animals, including the controls, were maintained on a regular diet (McCollum) and were housed in heated cages.

Blood for this study was obtained by heart puncture under aseptic conditions, 0.2 cc. of blood being removed. No anesthesia of any kind was used while securing blood samples. The blood so obtained was immediately placed in a small vial containing a minute amount of powdered heparin. This method was found to be very satisfactory. Blood was obtained six and seven times with no signs of injury.

Erythrocyte and total white cell counts were done with standard pipettes and the improved Neubauer counting chamber. Differential counts were done on dried smears made by the two-coverglass technic and stained with Wright's stain. Two hundred cells were counted for each determination. Reticulocyte counts were made on dried smears stained with a 0.5% alcoholic solution of cresyl blue and counterstained with Wright's stain. One thousand erythrocytes were counted and the reticulocyte count expressed as a percentage of this. Hemoglobin determinations were done on a Hellige hemometer so standardized that 14.5 gm. equals 100%.

RESULTS

From a study of 62 normal adult female rats of the age used in this experiment the normal erythrocyte count was found to be 8,260,000 per cu. mm. with a mean

deviation[4] of 680,000 cells per cu. mm.; the total white count 8,885 cells, with a mean deviation of 2,875; the reticulocyte percentage, 1.72% with a mean deviation of 0.98%; and a hemoglobin of 104% with a mean deviation of 4%.

To determine the possible effects on the blood picture of operative procedure and of anger and fear during handling incident to the blood removal, red and total white cell determinations were made on 13 'control operated' rats over a period of 70 days (fig. 1). The red count deviated around a mean of 8.26 million cells per cu. mm. with

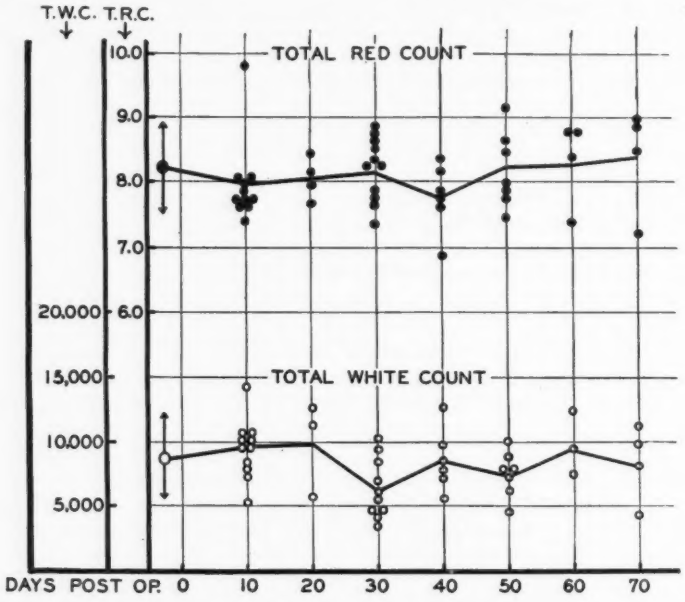

Fig. 1. Erythrocyte and total white cell counts of normal 'control operated' adult female rats. The lines represent averages.

no marked changes. The total white count which is known to be irregular under normal conditions showed no significant change. Neither the operative procedure nor the taming influence of handling had any effect upon the red and the total white cell count in the rat.

Hypophysectomy. The results of blood studies made after complete and incomplete hypophysectomy are shown in figure 2. The findings are reported separately for completely hypophysectomized animals and for animals showing microscopic fragments in the pituitary capsule.

After a postoperative rise, the erythrocyte count in completely hypophysectomized rats reached a definite anemic level of 5 to 6 million cells per cu. mm. 30 to 40 days after hypophysectomy. The incompletely hypophysectomized group also showed a postoperative rise. The amount of pituitary present, however, was not of sufficient quantity to prevent the occurrence of a definite anemia 40 days after operation. After a postoperative interval of 40 to 50 days the red cells in both groups reached a plateau, fluctuating around a level of 6 million cells.

The fall in the red count was accompanied by a distinct decrease in hemoglobin.

[4] As obtained by the formula:— $E = \sqrt{\sum d^2/N - 1}$.

Forty days after hypophysectomy both groups, those with and without pituitary fragments, showed a decrease from the control level of 104% to 75 and 80%. This was further lowered to 65 and 70% at 60 days after the operation.

Regardless of the completeness of the pituitary removal the total white count showed a rise from the control average of 8.8 thousand cells per cu. mm. to an average

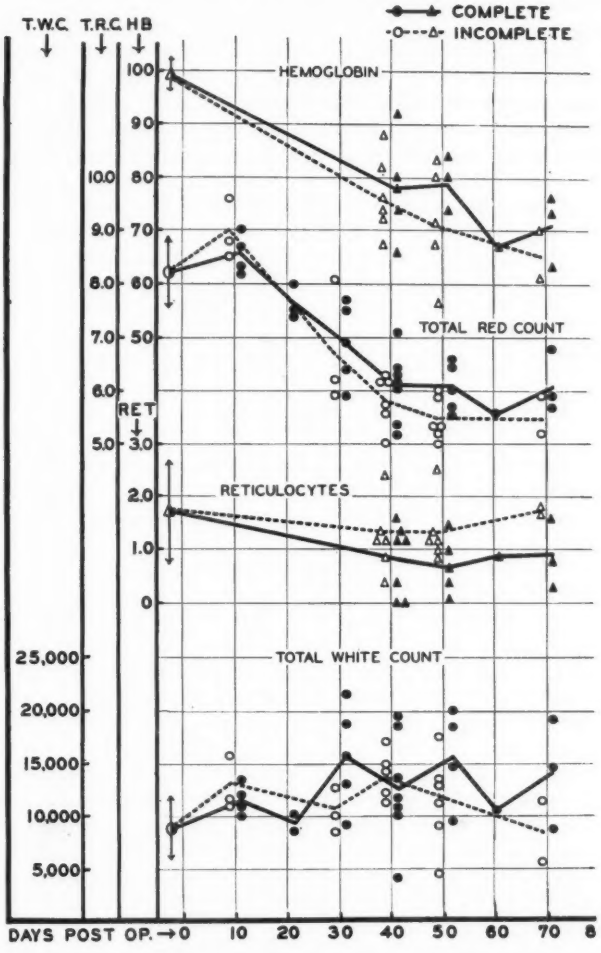

Fig. 2. Hemoglobin percentages, erythrocyte counts, reticulocyte percentages and total white cell counts in completely hypophysectomized adult female rats and in rats with microscopic fragments in the pituitary capsule. The lines represent averages.

of 15.8 thousand in the complete absence of the pituitary, and of 13.9 thousand in those rats exhibiting pituitary fragments. Because of the variability of these figures (fig. 2) they were treated statistically and were found to be significant. According to Fisher's table, there is a probability of 2 in 100 that these results were due to chance.

The averages of the reticulocyte counts showed a difference between the completely hypophysectomized animals and those with microscopic fragments. The complete group showed a drop from the normal average of 1.7% to below 1% while the incomplete group showed no change from normal levels.

The differential count (table 1), though showing a slight lymphocytosis, was essentially normal as was the fragility of the erythrocytes.

Hypophysectomy in these adult female rats produced a reticulocytopenia, an anemia, a decrease in hemoglobin, and a rise in the total white count. There was no significant change in the differential count or the fragility of the erythrocytes.

Adrenalectomy (+1% NaCl). A hemoconcentration is known to follow total adrenalectomy and the survival time at best is of short duration. Either of these factors would hide an anemia which might develop after adrenalectomy. In order to prevent

TABLE 1. SUMMARY OF DIFFERENTIAL COUNTS OF WHITE CELLS IN THE BLOOD OF FEMALE RATS

	Neutrophils	Eosinophils	Basophils	Lymphocytes	Monocytes
	%	%	%	%	%
Normal	17.0	2.6	0.1	77.8	0.7
Hypophysectomized	10.9	2.4	0.0	85.3	1.1
Adrenalectomized+1% NaCl	13.9	4.8	0.2	79.2	1.1
Thyroidectomized	33.8	2.6	0.07	60.4	3.6

this hemoconcentration and to prolong the survival time 1% NaCl was given to adrenalectomized adult female rats as drinking water. The results of blood studies made on rats after adrenalectomy with subsequent maintenance on 1% NaCl are shown in figure 3.

The red count in this group decreased in 10 to 20 days from the normal average of 8.26 million cells to a count of 6 million cells, almost the exact level attained by the hypophysectomized animals in 30 to 40 days. This level was not maintained, however, for there was a gradual return to the normal condition. Fifty days after operation an average count of 7.5 million cells was found. A group of normal adult female rats given 1% NaCl in a similar manner developed no anemia (fig. 3).

The hemoglobin content of the blood of rats adrenalectomized and subsequently maintained with 1% NaCl decreased from the normal of 104 to 67% in 10 days. This is also the level attained by the hypophysectomized animals. However, 20 days after the operation the hemoglobin level returned to slightly less than the normal values, and this level was maintained for as long as the animals were studied. This return of the hemoglobin to nearly normal values was more rapid than the return of the red count. At 20, 30, and 40 days after operation an anemia of 6.05, 6.59, and 6.93 million cells was accompanied by hemoglobin values of 89, 94, and 92%. This resulted in a color index greater than 1.

The reticulocyte percentage increased from the control level of 1.7 to a high of 2.8%. There was no decrease in reticulocyte percentages at any time.

Adrenalectomy followed by maintenance with NaCl had no effect on the total white count, the differential count, or the fragility of the erythrocytes.

Thus, adrenalectomy in these rats followed by 1% NaCl as drinking water produced an anemia, a reticulocytosis, a temporary decrease in hemoglobin, and a color index greater than 1. It had no effect upon the total white count, differential, or on the fragility of the erythrocytes.

Thyroidectomy. Thyroidectomy resulted in a moderate anemia, the erythrocyte

count decreasing gradually from the normal of 8.26 million cells to 6.47 million in 90 days (fig. 4). The hemoglobin decreased slightly, a nearly normal level being maintained during the period of study and resulting in a color index greater than 1.

The reticulocyte percentage remained normal after removal of the thyroid.

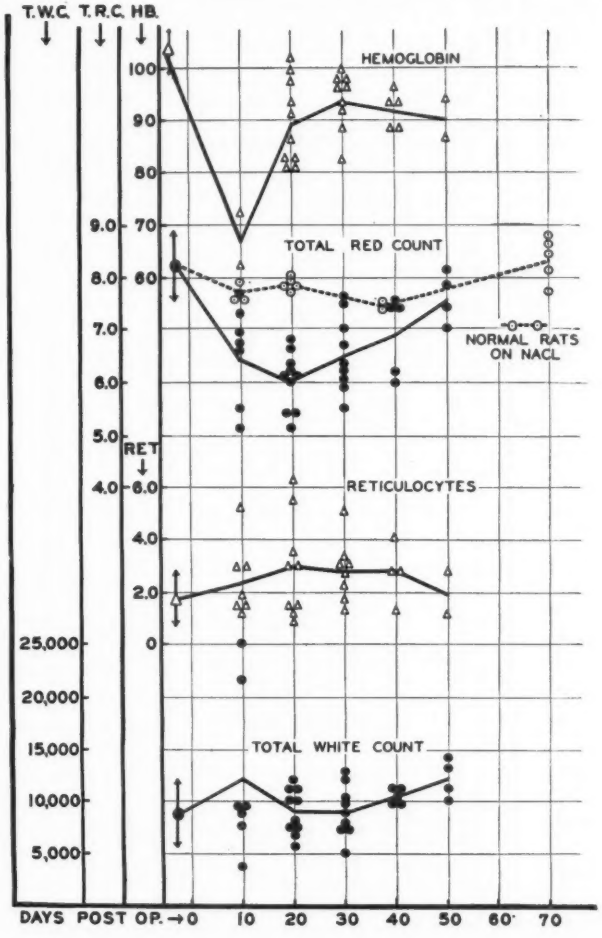

Fig. 3. HEMOGLOBIN PERCENTAGES, ERYTHROCYTE COUNTS, RETICULOCYTE PERCENTAGES AND TOTAL WHITE CELL COUNTS IN ADRENALECTOMIZED ADULT FEMALE RATS. These rats were maintained with 1% NaCl. The lines represent averages.

The total white count remained normal but differential counts showed a relative lymphocytopenia and an increase in neutrophils. Fragility tests showed the erythrocytes reacting normally.

Thus, after thyroidectomy in these adult female rats there was found a moderate anemia, a normal reticulocyte percentage, a normal total white count, a normal fragility, a slight decrease in hemoglobin, and a color index greater than 1.

DISCUSSION

In order to demonstrate that the hypophysis exerts its influence upon erythropoiesis secondarily through the thyroid or the adrenal glands, three steps are necessary. (*a*) The removal of either the thyroid or the adrenal glands should produce the

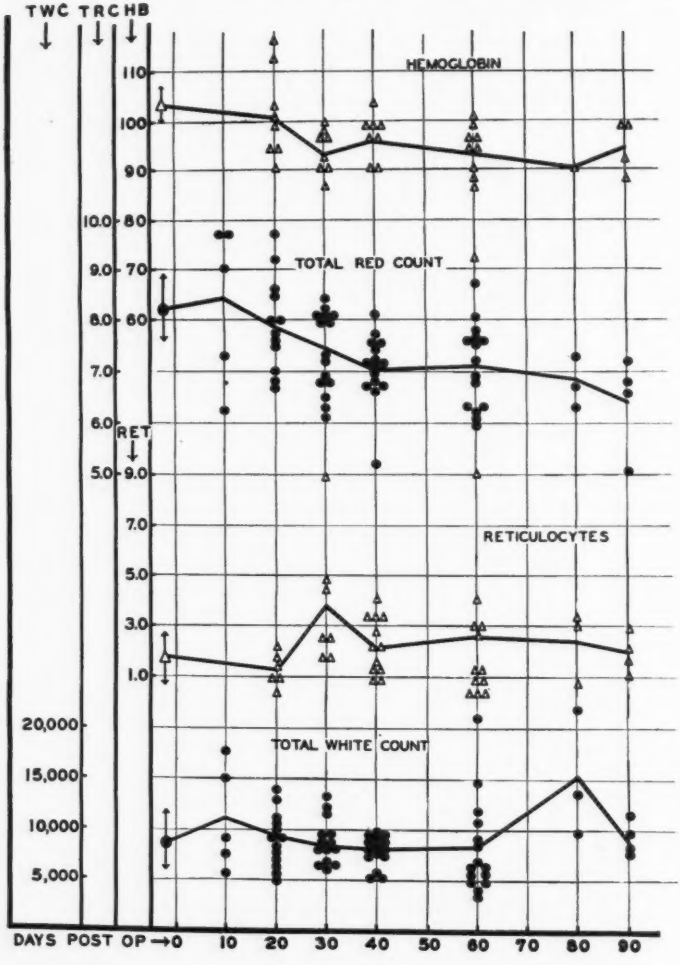

Fig. 4. HEMOGLOBIN PERCENTAGES, ERYTHROCYTE COUNTS, RETICULOCYTE PERCENTAGES AND TOTAL WHITE CELL COUNTS IN THYROIDECTOMIZED ADULT FEMALE RATS. The lines represent averages.

same results as the removal of the hypophysis. (*b*) The hormones produced by either the thyroid or the adrenal glands should prevent the symptoms caused by the removal of the hypophysis. (*c*) A pituitary preparation should not be able to stimulate erythropoiesis in the absence of the thyroid or the adrenal glands.

The present report is a comparison of the effects obtained on the cellular blood picture by hypophysectomy, adrenalectomy (+1% NaCl), and thyroidectomy. The

effects of thyroidectomy, and adrenalectomy, with subsequent maintenance with 1% NaCl, upon the cellular blood picture of these adult female rats differ in many respects from those found after hypophysectomy. The loss of hemoglobin, the decrease in erythrocytes, and the reticulocytopenia obtained after hypophysectomy point to a lack of capacity of the bone marrow to produce erythrocytes. On the other hand, the anemias following thyroidectomy and adrenalectomy (+1% NaCl) are accompanied by signs of an active bone marrow in that the normal reticulocyte percentages are maintained even though the anemia in adrenalectomized rats is of a severity comparable to that in hypophysectomized rats. In addition, the hemoglobin after thyroidectomy and adrenalectomy (+1% NaCl) in spite of a sharp initial drop, is maintained at nearly normal levels. The adrenalectomized animals, however, were maintained with 1% NaCl. There is a remote possibility that NaCl therapy might in some way stimulate the bone marrow and also the formation of hemoglobin.

It is possible that 1% NaCl might cause a hemodilution in adrenalectomized animals in spite of the finding that it did not cause an anemia in normal rats (fig. 3). In fact, water balance must be taken into account when evaluating the effects upon the cellular blood picture of removal of any endocrine gland. Since total plasma volume determinations are practically impossible in the rat total protein values have been used as an indicator of plasma volume. Levin and Leathem (35) studied the effects of the removal of the hypophysis, the thyroids, and the adrenals upon the blood chemistry of the rat. These authors found very little change in the total serum protein after any of these operations. They did point out that the albumin was decreased and the globulin increased which altered the AG ratio. Under these circumstances total protein figures as an indicator of plasma volume are unreliable. The gradual rise of the red count to normal levels in the adrenalectomized animals may be due to accessory gland tissue or to the fact that 1% NaCl will not maintain adrenalectomized animals indefinitely (36–39). Further investigations on the anemia produced by adrenalectomy with subsequent maintenance with 1% NaCl are in progress.

A change in water balance would not seem to be the factor responsible for the anemia following hypophysectomy. The effects found in the bone marrow (14) and in the reticulocyte percentage, both uninfluenced by water balance, a high total white count, the inability of the rat to respond to low oxygen tension with the typical bone marrow hyperplasia, increase in reticulocytes, and the red count of normal rats under similar conditions (12) tend to discount the possibility of hemodilution as a cause for the anemia of hypophysectomy.

The data presented in figure 1 eliminates the influence of taming as a cause for the decrease in the red count. Those in figure 2 show that the withdrawal of blood from an animal, which possibly cannot replace the loss, is not the cause of the anemia. Animals hypophysectomized for 40 days with no blood loss prior to that day show the same degree of anemia as those from which the blood has been removed every 10 days following hypophysectomy.

Meyer, et al. (13) placed emphasis upon the fact that the reticulocytes decreased below 1% if the animal was completely hypophysectomized but that such a reticulocyte percentage was not necessarily an indication of hypophysectomy. Vollmer, et al. (14), on the other hand, reported a similar drop in the reticulocyte percentage but claimed that this percentage is not reliable for they obtained an increase to 5% in one animal weeks after hypophysectomy. The data shown in figure 2 would seem to substantiate the findings of Meyer but examination of the individual points (fig. 2) shows a few normal reticulocyte percentages in the completely hypophysectomized animals. In the completely hypophysectomized group 62.5% of the reticulocyte determinations were 1% or less while in the group which had microscopic fragments 28.6%

had a similar low reticulocyte count. It would seem that the reticulocytes are very sensitive and will respond to the presence of microscopic fragments of the pituitary gland tissue.

The reticulocyte percentage will drop from other causes, as was shown by Fisch and Emmel (40), Smith and Gross (41), and Alt (42). The first authors found that the reticulocyte percentage and the percentage of polychromasia would decrease to .01 and 0% respectively with 132 hours of complete inanition. Smith and Gross, and Alt also found a correlation between growth and nutrition and the percentage of reticulocytes. Control of the diet so that growth was retarded decreased the number of reticulocytes. This would lead one to suspect the decreased food intake and the decreased growth of the hypophysectomized animals to be a cause for the anemia. Smith and Gross (41) and Alt (42) both found, however, that though there was a drop in reticulocyte percentage, there was no decrease in the red count. Vollmer, et

TABLE 2. SUMMARY OF EFFECTS OF GLAND EXTIRPATION ON BLOOD CELL COUNTS IN THE ADULT FEMALE RAT

	Hypophysectomy	Adrenalectomy (+1% NaCl)	Thyroidectomy
Reticulocyte percentage	decreased	increased	slightly increased
Red count	marked anemia	anemia	anemia
Hemoglobin percentage	decreased	temporary decrease→normal	slightly decreased
Color index	normal	greater than 1	greater than 1
Total white count	slightly increased	normal	normal
Differential count	slight lymphocytosis	normal	neutrophilia+lymphopenia
Fragility	normal	normal	normal

al. (14) found no anemia which was comparable with that following hypophysectomy when the food intake of normal rats was reduced to the level of that of the hypophysectomized animals. In addition, Alt found that the stunted animals responded to bleedings with a reticulocyte rise just as did the normal rats. The hypophysectomized rats would seem to be unable to respond in such a manner (12).

Reduction of the food intake or control of the diet so that growth is retarded does not necessarily duplicate the conditions existing in the hypophysectomized animals. It may not be food intake but food utilization which is important. The upset in metabolism may still be the cause of the anemia found after hypophysectomy.

A suggestion that the pituitary may regulate blood destruction rather than blood formation is seen in the work of Querido and Overbeck and in the work of Dodds and co-workers. The latter group, in a series of papers (43–45), claim that the anemia resulting from posterior lobe extract administration was due to an increased blood destruction. McFarlane and McPhail (46) also reported an anemia with injection of similar substances. Gilman and Goodman (47–49), however, claim the anemia had nothing to do with blood destruction but was simply a water retention. If posterior lobe extracts will cause an anemia by increasing the rate of blood destruction one would not expect an anemia to follow the removal of the posterior lobe of the pituitary. In addition, the fragility of the erythrocytes remains normal and the spleen decreases in size (50) after hypophysectomy.

The cause for the anemia following the removal of the pituitary is still problematical, and any statement as to whether the hypophysis exerts its influence upon erythropoiesis directly or secondarily through the atrophy of the thyroid or adrenal glands must await further work upon the effect on the cellular blood picture of thyroid and adrenal gland hormones in the hypophysectomized rat and the effect of a suitable pituitary preparation in the thyroidectomized and adrenalectomized rat.

SUMMARY

The data obtained from blood studies following hypophysectomy, thyroidectomy, and adrenalectomy with subsequent maintenance with 1% NaCl in these adult female rats is summarized in table 2. Under the conditions of this experiment, the anemia following hypophysectomy is associated with indications of inactive erythropoiesis while the anemias after thyroidectomy and after adrenalectomy (+1% NaCl) are associated with indications of a normal or increased erythropoiesis.

Acknowledgment is gratefully made to Dr. Earl T. Engle for his criticisms and suggestions in connection with this study.

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THE EFFECT OF ENDOCRINES ON THE FORMED ELEMENTS OF THE BLOOD[1,2]

II. The Effect of Estrogens in the Dog and Monkey

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Large doses of estrogens exert a profound influence upon the cellular blood picture of some species of animals. In an attempt to lower the serum Ca level of dogs with large doses of estrogens, Arnold and coworkers (1, 2) found that large doses interfered with hemopoietic processes to such an extent that death resulted. The hemorrhagic purpura, gradual anemia and leucocytosis followed by a leucopenia found by these authors in the dog was soon confirmed by three groups of European workers (3, 4, 5). Tyslowitz (6), Arnold (7), and McBride (8) repeated the above experiments using stilbestrol and obtained similar results.

Because of the clinical significance of such findings it seemed to be of importance to extend this work to a species which is phylogenetically closer to man. This study was undertaken to confirm the work done upon the dog and to extend the observations to the rhesus monkey.

While this work was in progress, Tyslowitz (9) studied the monkeys in the Carnegie colony in Baltimore and stated that "the blood picture suffered little change" when these animals were treated with large doses of estrogens.

METHODS

The animals used in this experiment were adult female dogs and monkeys. The dogs were trained for a period of a week or longer before blood samples were taken.

Table 1. Differential blood counts in the normal adult female dog

Dog	No. of counts	Neutrophils	Eosinophils	Basophils	Lymphocytes	Monocytes	Other types
		%	%	%	%	%	%
1	4	73.9	4.5	0.4	11.2	7.7	2.2
2	2	65.0	3.0	0.0	22.0	7.0	3.0
3	2	71.0	6.5	0.0	14.0	2.5	1.0
4	6	72.5	1.9	0.3	23.9	1.9	1.0
5	3	59.0	6.3	0.0	24.3	5.0	5.3
Average %		68.3	4.4	0.01	19.1	4.8	2.5

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[1] Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, in the Faculty of Pure Science, Columbia University. For Part I, see preceding paper, The Effects of Hypophysectomy, Thyroidectomy, and Adrenalectomy on the Blood of the Adult Female Rat.

[2] Aided by a grant from the Rockefeller Foundation, administered by Dr. P. E. Smith.

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One half cc. of blood was removed from the external jugular vein after a rest period of 10 to 15 minutes during which time the dog was flat on its back in a special dog board. In this manner the blood was taken under conditions of maximum rest. The monkeys were impossible to train in the above manner. In order to approach a condition of

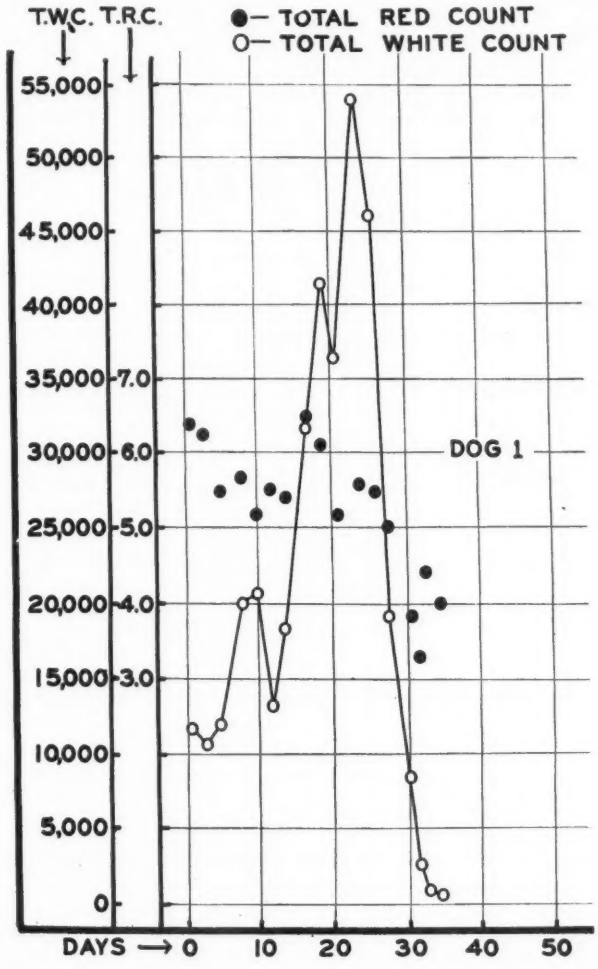

Fig. 1. Erythrocyte and total white cell counts in *dog 1* injected with 5 mg. of stilbestrol per day for 27 days followed by 10 mg. per day for 7 days. Death occurred on the 34th day.

rest, they were placed in burlap bags for 20 to 30 minutes before blood was removed from the small saphenous vein. Anesthesia was not used for obtaining blood samples and all blood was removed at the same hour of the day. The monkeys had been given tuberculin tests and were found to be free of this disease.

Red cell counts, total white cell counts, hemoglobin percentages, and differential counts were made on heparinized blood. Erythrocyte and total white cell counts

were done with standard pipettes and the improved Neubauer counting chamber. Differential counts were made on dried smears prepared by the two-coverglass technic and stained with Wright's stain. Hemoglobin determinations were made on a Hellige hemometer so standardized that 14.5 gm. equals 100%.

RESULTS AND DISCUSSION

Dogs. To determine the normal erythrocyte and total white cell count in the adult female dog, 3 animals were studied (*dogs* 1, 4, and 5) for 2 months during which time 18, 27, and 25 blood samples were studied respectively. These adult female dogs of 9

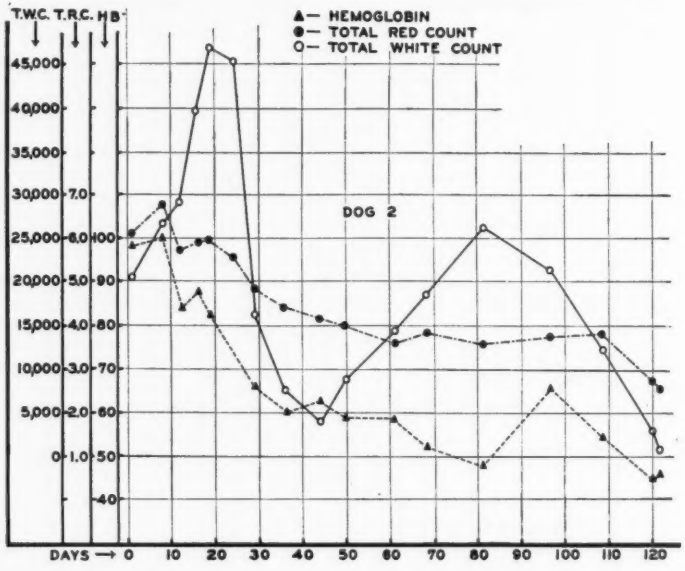

Fig. 2. Erythrocyte counts, total white cell counts and hb.% in *dog* 2 injected with 5 mg. of stilbestrol per day for 63 days followed by 10 mg. for 59 days. Death occurred on 122nd day.

to 10 kg. body weight had an erythrocyte count fluctuating around a level between 6 and 7 million cells per cu. mm. *Dog* 1 had a total white count fluctuating at a level between 10 and 15 thousand cells per cu. mm. *Dog* 4 settled down to this same level after a temporary rise during an estrous period. *Dog* 5 had a lower total white cell count of 5 to 10 thousand cells. Differential counts were made on 5 dogs and these are summarized in table 1.

These figures agree with those given in a review of the literature by Scarborough (10).

Dogs 1, 2, and 3 were treated with stilbestrol. The details of the results obtained are presented in figures 1, 2, and 3. In *dog* 1 (8 kg. body weight), 5 mg. of stilbestrol in oil per day for 27 days followed by a daily dose of 10 mg. for 7 days resulted in a sharp rise in the total white cell count to a peak of 54.3 thousand cells per cu. mm. in 23 days. This was followed by a rapid drop to 200 cells per cu. mm. by the 34th day, the day of death. The erythrocyte count gradually decreased from 6.4 million

cells to 4 million cells. Thrombocytopenic hemorrhagic purpura started on *day* 25 and proestrous bleeding occurred on *day* 23.

Dog 2 (8.0 kg. body weight) received 5 mg. of stilbestrol intramuscularly per day for 63 days followed by 10 mg. per day for 59 days. The total white count showed a rise in 18 days to 46.9 thousand cells per cu. mm. which was followed by a drop to 4 thousand cells on the 43rd day of treatment. Unlike *dog* 1, the total white count showed a second rise to 26 thousand cells which was again followed by a decrease

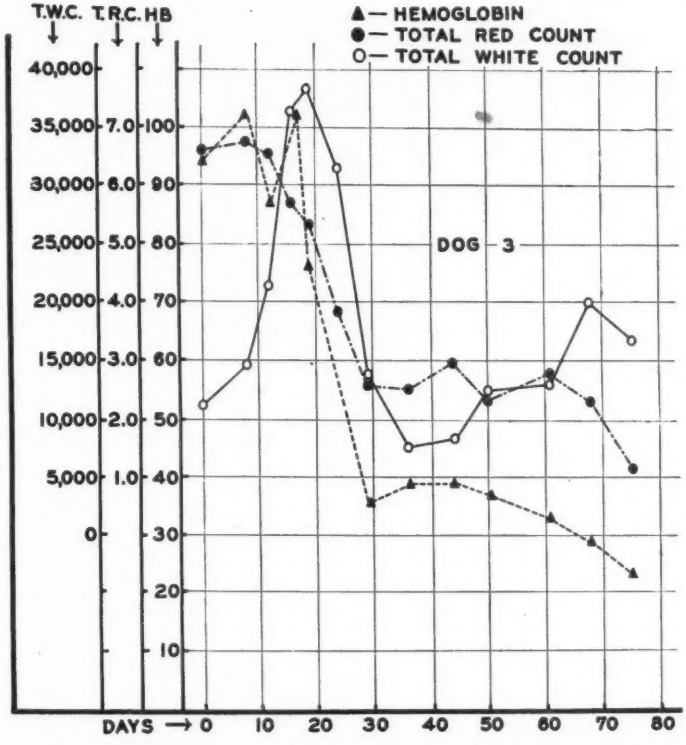

Fig. 3. Erythrocyte counts, total white cell counts, and hb.% in *dog* 3 injected with 10 mg. of stilbestrol per day. Death occurred on the 75th day.

to 850 cells per cu. mm. on the 122nd day, the day of death. The red count decreased from the control level of 6.1 million cells to 2.6 million cells during this treatment and the hemoglobin dropped from 98 to 47% in the same period. Thrombocytopenic hemorrhagic purpura started on *day* 28.

Dog 3 (12.0 kg. body weight), received daily injections of 10 mg. of stilbestrol in oil per day. The total white count increased from the normal of 11 thousand cells per cu. mm. to 38.1 thousand cells on the 18th day of treatment and then decreased to 7.5 thousand cells on *day* 36. This was followed by a subsequent rise to 19.5 thousand cells and a normal count of 15.2 thousand cells was found on the 75th day of treatment, the day of death. The red count decreased from 6.5 million cells to

1.1 million and the hemoglobin decreased from 95 to 23%. Thrombocytopenic hemorrhagic purpura started on *day* 43 and proestrous bleeding occurred on *day* 8.

Differential counts on these 3 animals (table 2) demonstrated that the rise in the total white count was due to a rise in the number of neutrophils. These showed an increase in the % of young forms during the early days of the treatment and a return to normal followed by a shift to the right in the later days of treatment. If the blood showed a secondary rise in the total white cell count (*dogs* 2 and 3), the percentage of neutrophils again increased.

TABLE 2. DIFFERENTIAL BLOOD COUNTS OF NORMAL ADULT FEMALE DOGS TREATED WITH INTRAMUSCULAR INJECTIONS OF STILBESTROL

Dog	Daily dose	Day of treatment	Neutrophils	Eosinophils	Basophils	Lymphocytes	Monocytes	Other types
	mg.		%	%	%	%	%	%
1	5	0	73.9	4.5	0.4	11.2	7.7	2.2
		8	81.5	1.5	0.0	1.5	15.0	0.5
		17	86.0	3.0	0.0	4.5	6.0	0.5
		21	90.5	1.0	0.0	5.0	2.0	1.5
		24	93.0	1.0	0.0	4.5	0.5	1.0
		28	95.0	0.0	0.0	4.0	0.0	1.0
		32	49.0	10.0	0.0	28.0	0.0	13.0
		35	Not enough cells to count					
2	5	0	65.0	3.0	0.0	22.0	7.0	3.0
		16	78.0	4.0	0.0	7.0	6.0	5.0
		24	89.0	1.0	0.0	7.0	3.0	0.0
		50	44.0	1.0	0.0	48.0	4.0	3.0
		61	44.0	1.0	0.0	45.0	9.0	1.0
	10	68	75.0	1.0	0.0	23.0	1.0	0.0
		96	77.0	2.0	0.0	12.0	6.0	3.0
		108	59.0	4.0	0.0	32.0	1.0	4.0
		122	Not enough cells to count					
3	10	0	71.0	6.5	0.0	14.0	2.5	1.0
		16	90.0	0.0	0.0	9.0	1.0	0.0
		24	96.0	0.0	0.0	4.0	0.0	0.0
		50	83.0	0.0	0.0	17.0	0.0	0.0
		68	93.0	0.0	0.0	7.0	0.0	0.0

Dogs 4 and 5, both weighing 10 kg. were injected with 5 mg. of alpha and beta estradiol[4] respectively per day. The injection of the 2 substances gave similar results except for the fact that the alpha estradiol raised the total white cell count to 79.8 thousand cells while the beta preparation raised the count to 56.4 thousand cells. There was an increase in the percentage of young cell forms followed by a return to normal percentages. A slight anemia resulted, both preparations causing a drop from 6.00 to 7.00 million cells to 4.00 to 5.00 million cells. Neither hormone caused the death of the animal, although thrombocytopenic hemorrhagic purpura developed on the 41st day with the beta estradiol and on the 47th day with the alpha estradiol.

As neither *dog* 4 nor *dog* 5 died from the above treatment, 5.0 mg. of stilbestrol were injected per day for 50 and 103 days respectively. Neither dog responded to

[4] Beta estradiol was procured in two batches. The first was obtained by courtesy of Dr. Erwin Schwenk in a form contaminated by estrone and alpha estradiol. The latter two hormones were removed by use of Girard's Reagent and repeated crystallizations. The resulting product melted at 220°. The second batch was obtained by the courtesy of Dr. Oskar Wintersteiner. This preparation was a slightly impure form with a melting point of 221° C. Pure beta estradiol melts at 223 degrees (11).

these injections in a manner similar to that of the initial treatment. *Dog 4* showed no change in the cellular blood picture. The animal became emaciated in spite of a good appetite, lost most of its hair, had difficulty in walking, and had a bad odor. *Dog 5* developed similar symptoms but to a less degree.

Examination of the differential counts given in table 3 shows that both of these animals responded with an increase in the neutrophils and a relative decrease in the lymphocytes. These dogs receiving alpha and beta estradiol under the conditions of this experiment did not show a neutrophil count below normal even after extensive secondary treatment with stilbestrol.

These dogs responded to large doses of estrogen with an initial marked neutro-

TABLE 3. DIFFERENTIAL BLOOD COUNTS OF NORMAL ADULT FEMALE DOGS TREATED WITH ALPHA AND BETA ESTRADIOL FOLLOWED BY INTRAMUSCULAR INJECTIONS OF STILBESTROL

Dog	Daily dose	Day of treatment	Neutrophils	Eosinophils	Basophils	Lymphocytes	Monocytes	Other types
	mg.		%	%	%	%	%	%
4	5	0	72.5	1.9	0.3	23.9	1.9	1.0
	alpha	9	80.5	3.5	0.0	9.0	6.0	1.0
	estradiol	13	87.5	0.0	0.0	8.5	4.0	0.0
		18	87.5	0.5	0.0	8.5	3.0	0.5
		23	93.0	2.0	0.0	3.5	1.0	0.5
		25	93.5	0.0	0.0	4.5	2.0	0.0
		27	94.5	0.5	0.0	5.0	0.0	0.0
		31	84.0	1.5	0.0	9.5	0.5	4.5
		72	83.5	1.0	0.0	10.5	3.0	2.0
	5	75	Stilbestrol started					
	stilbestrol	83	77.0	4.0	0.5	14.0	1.5	3.0
		104	89.5	0.0	0.0	8.5	2.0	0.0
		125	83.5	0.0	0.0	12.0	4.0	0.5
5	5	0	59.0	6.3	0.0	24.3	5.0	5.3
	beta	19	80.0	6.0	0.0	10.0	3.0	1.0
	estradiol	22	84.0	5.5	0.0	7.0	3.5	0.0
		26	83.0	6.0	0.0	6.5	3.0	1.5
		31	97.5	1.0	0.0	1.5	0.0	0.0
		34	89.0	3.5	0.0	4.0	2.5	1.0
		41	80.5	4.0	0.0	13.0	2.5	0.0
		58	84.5	1.0	0.0	10.5	1.0	3.0
		120	64.0	13.0	0.0	14.5	4.0	1.0
	5	121	Stilbestrol started					
	stilbestrol	215	69.0	0.0	0.0	31.0	0.0	0.0

philia followed by a neutropenia, an increase followed by a return to normal or a decrease in young forms, an anemia, and a decrease in hemoglobin. These primary changes were constant for 5 dogs, but the terminal effects varied in each animal. After a single rise in the number of white cells *dog 1* had an extremely low total white count figure at death and a moderate anemia while *dog 2* showed a double rise in the total white count and at death had a low white count and a severe anemia. *Dog 3* showed a double elevation of the white count but had a severe anemia and a normal total white count at death. *Dogs 4* and *5* showed a single rise in the total white count and they continued to live in spite of continued injections of the hormone. The hematopoietic system of the latter 2 dogs did not respond to a different estrogen in a manner similar to the initial effect. Death from estrogen injections seemed to depend in part upon the physical condition of the animal and upon sufficient dosage.

Monkeys. The determination of normal figures for the cellular content of the blood of monkeys is difficult. Table 4, a summary of the literature on this subject, shows the marked variation in the total white cell count and the differential count obtained by various workers. The difference between an average count of 5.2 thousand cells per cu. mm. (16) and 26.5 thousand cells (22) shows the extreme variability in the total white count which may be obtained from the same species of monkey. The percentages reported for the neutrophils and the lymphocytes, the two main components of the total white cell count, also show a marked variability. The extremes of 24 to 65% for the neutrophils, and 22 to 52% for lymphocytes (13) shows that the averages of 45 and 37% are of questionable significance.

TABLE 4. SUMMARY OF THE REPORTS IN THE LITERATURE ON THE TOTAL WHITE CELL COUNT AND THE DIFFERENTIAL COUNT OF MONKEY BLOOD

Authority	Total white count (thousands)			No. of counts	No. of animals	Differential counts	
	Av.	Max.	Min.			Neutrophils	Lymphocytes
						%	%
Anderson, Neill (12)	11.2	12.6	8.3	100	10	42 (32–50)	54 (38–63)
Hektoen, Eggers (13)	18.5	23.9	15.5	4	4	45 (24–65)	37 (22–52)
Kleineberger, Carl (14)	7.4				6	35.8	58
Sellards, Boetizer (15)	25.0					40	60
Wells, Sutton (16)	5.2						
Taylor (17)	22.2			121	40	41	54
Taylor (18)				4	4	38 (34–41)	58 (54–63)
Krumbhaar, Musser (19)	13.4 14.2	15.0	8.9	7 15	7 12	47	46
Fox (20)	11.5	25.0	5.0			(44–54)	(33–47)
Shukers (21)		20.5	9.7			(20–52)	(44–74)
Lucus, Prizer (22)	26.5	31.6	23.6	3	3	(45–52)	(27–48)

Because of the marked variability in the reports of the blood picture in the monkey, 3 animals (*618*, *625* and *627*) were studied for 3 months; 29, 27 and 26 blood samples being examined respectively in an attempt to establish normal values for the cellular blood picture in the rhesus monkey in the absence of anesthesia. The 3 monkeys maintained an erythrocyte count of fair consistency. *Monkeys 618* and *625* had a count ranging between 5 and 6 million cells while *monkey 627* exhibited a count at a slightly lower level of 4.3 to 5.3 million cells (fig. 4). These counts agree with the average of 90 counts by 4 authors reported by Scarborough (10) as 5,590,000 cells.

The total white cell count of *monkey 618*, the more tractable of the 3 animals started with a range of 12 to 19 thousand cells per cu. mm. but soon settled down to a level of 10 to 15 thousand cells. *Monkey 625* started with a total white count of 26.5 and gradually decreased in 30 days to the level of 10 to 15 thousand cells, the

same level as was found in *monkey 618*. *Monkey 627*, a particularly intractable animal, started with a total white count of 34.6 thousand cells. During the following 2½ months the count gradually decreased to 20 to 25 thousand cells. As there was little decrease in the pugnacity of the animal during this period the monkey was considered unsuitable for further blood work. Another intractable animal, *monkey 606*,

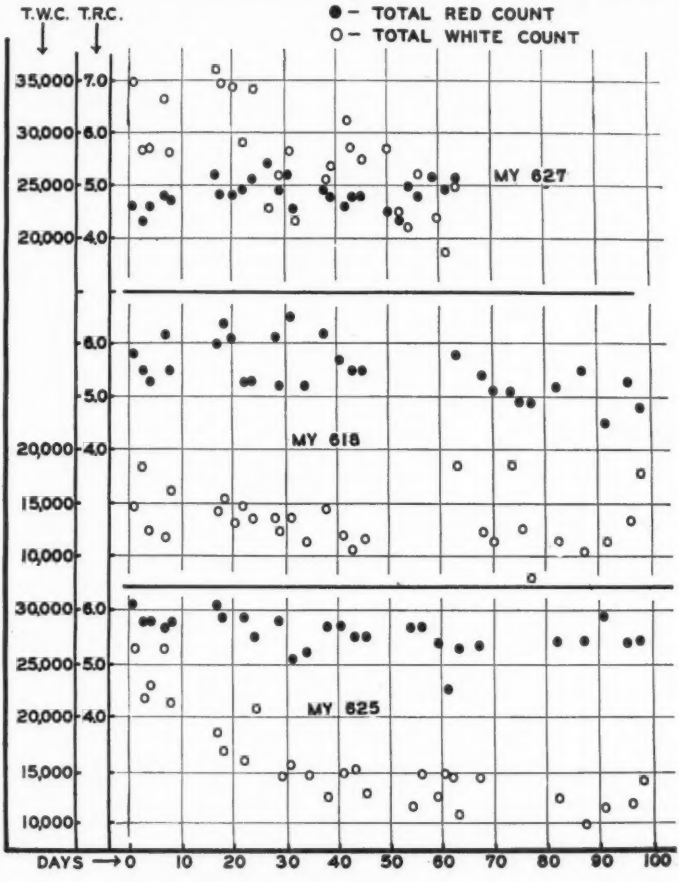

Fig. 4. Erythrocyte and total white cell counts of *monkeys 627, 618* and *625* during a control period of no treatment.

was examined and exhibited a total white cell count of 42.7 thousand cells per cu. mm. This animal also was not used for this work.

Thus 4 monkeys showed total white cell counts of 14.5, 26.5, 34.6, and 42.7 thousand cells per cu. mm. on first study. Upon further study of the 2 animals which were most easily handled a base line for white blood cells was established between 10 and 15 thousand cells per cu. mm. (fig. 4).

A study of the differential counts showed that the increase in the total white count was due to an increase in circulating lymphocytes. *Monkey 618*, with an initial

total white count of 14.5 thousand cells, had 67.5% lymphocytes and 30% neutrophils; *monkey 625* with an initial count of 26.5 thousand cells, had 67% lymphocytes and 29.5% neutrophils; and *monkey 627* with a total white count of 34.6 thousand cells, had 92% lymphocytes and 7.5% neutrophils. As can be seen in table 4, the percentage of lymphocytes gradually decreased and the percentage of neutrophils increased as the 2 animals became accustomed to the routine of taking blood samples. After a control period of one month for *monkey 618* and 3½ months for

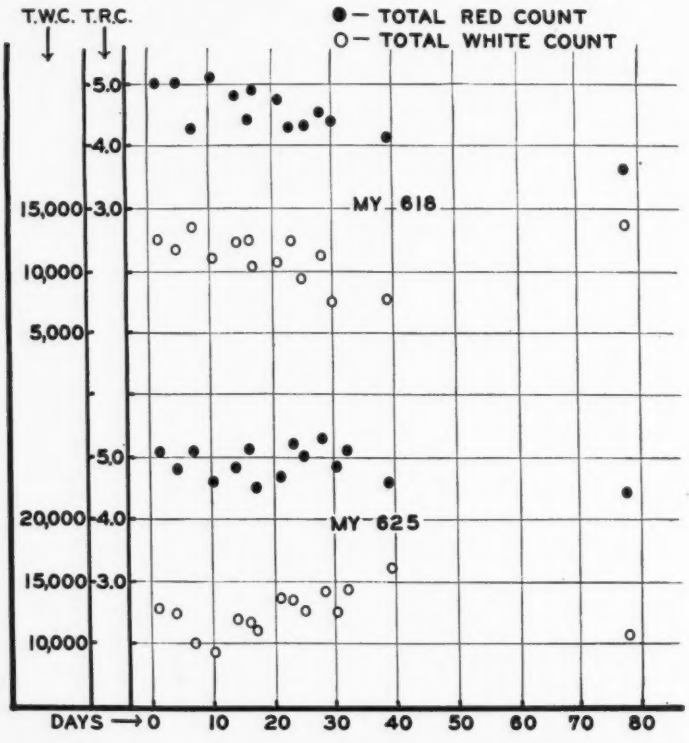

Fig. 5. Erythrocyte and white cell counts in *monkey 618* treated orally with 10 mg. of stilbestrol and in *monkey 625* injected intramuscularly with 10 mg. of stilbestrol per day.

monkey 625, during which time no treatment was given, the predominance of cells became reversed so that the differential count resembled that for the dog and human being. From these data the basis for the discrepancies in the values reported in the literature becomes apparent. Further work on a greater number of animals in a condition of rest is essential to establish a normal differential blood count for the rhesus monkey.

These data established that the red count in 3 monkeys was between 5 and 6 million cells per cu. mm. and the total white count was 10 to 15 thousand cells. The data show that blood counts on a small series of monkeys which are conditioned to handling and are studied thoroughly are more significant than counts on a large series of animals studied without conditioning or regard to states of fear or anger.

The probable cause for the rise in the white cell count is the fear and muscular tension developed in the monkey when it is being caught. It has been demonstrated repeatedly that a contraction of the spleen, with an outpouring of lymphocytes and erythrocytes, takes place under the influence of fear (23, 24). It has been stated (25, 26) that an increased number of lymphocytes enter the subclavian vein when there is increased muscular tension producing a massage of the lymph channels.

Two adult female monkeys, whose blood figures had been previously determined,

TABLE 5. DIFFERENTIAL COUNTS OF NEUTROPHILS AND LYMPHOCYTES IN THE BLOOD OF 2 RHESUS MONKEYS DURING A CONTROL PERIOD WITH NO TREATMENT

	Date	Neutrophils	Lymphocytes	Date	Neutrophils	Lymphocytes
		%	%		%	%
Monkey 618	3/ 9	30.0	67.5	4/ 8	41.5	46.0
	3/11	54.0	42.5	4/15	57.5	33.5
	3/12	42.5	52.5	4/20	56.5	30.5
	3/15	32.0	61.0	5/10	53.5	39.5
	3/16	40.0	52.0	5/15	57.0	37.5
	3/25	24.0	69.5	5/17	52.0	46.0
	3/26	25.5	71.5	5/22	57.5	35.5
	3/28	25.5	69.0	5/29	52.6	41.5
	3/30	33.0	62.0	6/ 7	54.0	40.0
	4/ 1	15.0	78.0	6/12	68.0	25.5
	4/ 5	30.5	57.5	6/14	60.5	36.0
Monkey 625	3/ 9	29.5	67.0	4/ 8	38.5	63.5
	3/11	20.0	77.5	4/11	37.5	55.0
	3/12	36.5	62.0	4/15	36.0	60.5
	3/15	43.5	54.5	4/18	33.0	65.0
	3/16	43.5	54.0	5/ 3	42.0	54.0
	3/25	31.5	68.5	5/14	35.0	61.6
	3/26	32.5	64.0	5/29	35.0	60.0
	3/30	32.5	62.0	6/ 3	33.0	61.1
	4/ 1	34.5	65.5	6/14	25.0	70.0
	4/ 6	36.0	59.0	6/19	55.0	40.5
				6/22[1]	65.5	31.0
				6/25	63.5	36.0
				7/ 2	38.5	59.0
				7/ 4	53.0	43.0
				7/ 9	63.0	35.5

[1] At this point injections were started. Because of the similar results in *monkey 618* with no treatment, it is questionable whether the injections had any effect on the differential counts.

were spayed and given 10 mg. of stilbestrol per day for 77 days. The results are shown in figure 5. *Monkey 618* received the 10 mg. dose orally while *monkey 625* was treated by intramuscular injections. There was a drop in the erythrocyte count from 5 million cells to 3.6 million in *monkey 618*, and from 5.2 million cells to 4.4 million in *monkey 625*. There was no significant change in the white cell count of either monkey. Study of the neutrophils showed no increase in the percentage of young forms.

Because of the marked response of the lymphocytes to any excitement, the differential counts obtained in monkeys were difficult to evaluate. The two main components of the differential counts, the neutrophils and the lymphocytes, showed a fluctuation in their proportion in the untreated monkey (table 5), so that the alteration of the percentages of these 2 types of cells in animals under estrogen treatment is of questionable significance. A report of the differential counts obtained in the monkeys is therefore omitted from this paper.

Recent work (27–32) indicates that the liver is the organ responsible for the inactivation of estrogens. It was thought that symptoms similar to those found in the dog as a result of estrogen treatment might be reproduced in the monkey if the action of the liver were partially or wholly removed. Accordingly, after a rest period of 2 months, these same monkeys were treated with CCl_4 in addition to the stilbestrol. The results are given in figure 6.

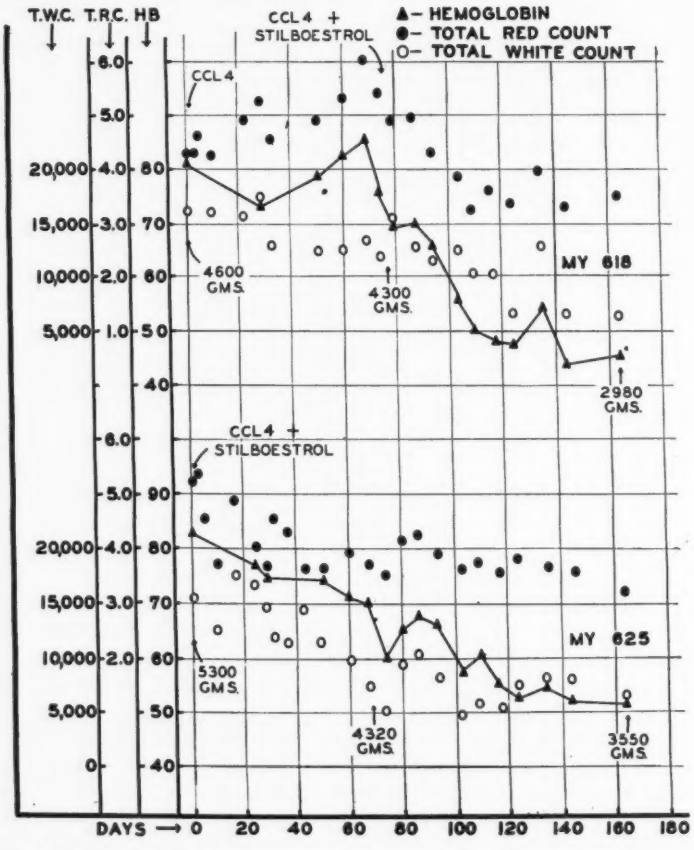

Fig. 6. Erythrocyte counts, total white cell counts and hb.% in *monkeys 618* and *625* receiving combinations of 1 cc. of CCl_4 per day orally and 10 mg. of stilbestrol per day intramuscularly.

In order to determine if CCl_4 would exert any influence upon the cellular blood picture, *monkey 618* was given 1 cc. of this material by stomach tube each day for 78 days. During this period the animal lost 300 gm. in weight but the cellular blood picture was not altered. In fact, the animal showed a gradual recovery from the last estrogen treatment. At the end of 78 days of CCl_4 treatment the monkey had a red count of 5 to 6 million cells, a total white count of 10 to 15 thousand cells, and a hemoglobin value of 80%, all normal figures. At this time 10 mg. of stilbestrol per

day were injected intramuscularly in addition to the CCl_4. After 30 to 40 days the erythrocyte count had decreased to between 3 and 4 million cells, the total white cell count to between 5 and 10 thousand cells, and the hemoglobin to between 40 and 50%. Another month and a half of treatment did not change these figures but did decrease the body weight to 2,980 gm. a total loss of 1,620 gm.

Monkey 625 received 1 cc. of CCl_4 by stomach tube plus 10 mg. of stilbestrol intramuscularly daily for 165 days. After 50 days of such treatment the total red count had decreased to 3 to 4 million cells and was not further depressed by this treatment. The hemoglobin percentage gradually decreased from 80% to between 50 and 60% and the total white count decreased from 10 to 15 thousand cells to between 5 and 10 thousand cells. The animal lost a total of 1,750 gm. during this treatment.

Examination of the neutrophils showed no evidence of an increase or decrease in the percentage of immature forms.

After treatment with 770 mg. of stilbestrol during a period of 77 days, the blood studies in these rhesus monkeys showed a slight anemia, no change in the total white cell count, and no change in the type of leucocytes. Even when a total dose of 1,650 mg. of stilbestrol was given simultaneously with 165 cc. of CCl_4 during a period of 165 days, the anemia, the leucopenia, and the decrease in hemoglobin were of moderate degree.

The dogs received a daily dose of ½ mg. of estrogen per kg. of body weight; the monkeys, 2 mg. of estrogen per kg. of body weight. Thus, the remarkable results obtained in the dogs could not be reproduced in monkeys with a daily dose of estrogen per kg. of body weight which was fourfold that used in the dogs.

SUMMARY

The figures for the cellular blood picture of normal adult female dogs of 9 to 10 kg. body weight are as follows. Erythrocyte count 6 to 7 million cells per cu. mm. Total white cell count 5 to 15 thousand cells per cu. mm.

neutrophils	68.3 %	lymphocytes	19.1%
eosinophils	4.4 %	monocytes	4.8%
basophils	0.01%	other types	2.5%

The figures for the cellular blood picture of spayed adult female rhesus monkeys of 4 to 5 kg. body weight are: erythrocyte count, 5 to 6 million cells per cu. mm., total white cell count 10 to 15 thousand cells per cu. mm. Because of the marked response of the lymphocytes to any excitement of the animal, differential figures were not established for the normal resting monkey.

Daily doses of 5 to 10 mg. of stilbestrol in dogs caused a marked neutrophilia followed by a neutropenia, an increase followed by a decrease in the young neutrophils, an anemia, and a decrease in hemoglobin. Daily doses of 5 mg. of alpha or beta estradiol produced similar results.

Except for a slight anemia, 10 mg. doses of stilbestrol in rhesus monkeys did not produce the conditions found in dogs with daily doses of 5 mg. of the same type of estrogen, although the monkeys received a fourfold dosage per kg. of body weight.

When the livers of the rhesus monkeys were damaged with CCl_4, an anemia, a decreased hemoglobin percentage, and a leucopenia were produced but to a moderate degree.

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ADENOFIBROMYOMATA IN THE UTERUS OF A GUINEA PIG AFTER EXTREME PARTIAL CASTRATION

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IT IS WELL KNOWN FROM THE WORK OF LIPSCHÜTZ (1), that extreme partial castration results in cystic glandular hyperplasia of the endometrium and atypical epithelial growth. Adult virgin guinea pigs initially weighing 300 to 400 gm. each were subjected to extreme partial castration in October 1939 for the purpose of studying the elimination of androgens and estrogens in the urine of such animals, as had already been done following injections of prolan (2). When the animals were killed after 14 months, one of them showed small apical tumors in both uterine horns.

This guinea pig weighed 710 gm. The vagina was closed; the nipples were similar to those of the other animals of the same lot. The uterus weighed 2.2 gm. The size of one of the tumors was equivalent to 2 to 3 and the others to 1 to 2 on the scale of Lipschütz (3).

Histological study. In the apical part of the right uterine horn on one side of the lumen there were two adenofibromyomatous growths and on the other side another fibromatous growth with a large cystic cavity (fig. 1). In the left horn there were 2 similar adenofibromyomatous growths. In figure 2 one of these is sectioned through its region of greatest diameter, the other almost at its lower pole. The growths were well encapsulated and lay between the muscle layers of the uterus. The uterine lumen appeared double (fig. 1), but in reality it was single, with a cystic cavity which in other sections was seen to communicate with the uterine cavity. There was also a glandular dilatation communicating with the uterine cavity (fig. 2). At other places in the uterus there were signs of cystic glandular hyperplasia, though these were not well marked.

Under higher magnification young connective tissue was seen surrounding the glands, and older connective tissue with smooth muscle fibers surrounded the glandular islets. In some places the glands were cystic and had flattened epithelium, but in other glands the epithelium appeared cuboidal (fig. 4).

In figure 3 the capsule of one of the tumors is well demonstrated. With Weigert's iron haematoxylin and picrofuchsin it was possible to distinguish the muscle fibers from the connective tissue and to show that the latter was present in greater proportion than the muscle fibers.

The small fragment of the ovary which remained after the operation contained a mature follicle of normal appearance and two follicles exhibiting thecal luteinization.

These findings were of interest since Lipschütz (3, 4, 6, 7) has not found uterine fibroids after extreme partial castration, but only cystic glandular hyperplasia and atypical epithelial growth.

Whether these tumors were present in the animal before operation, or were

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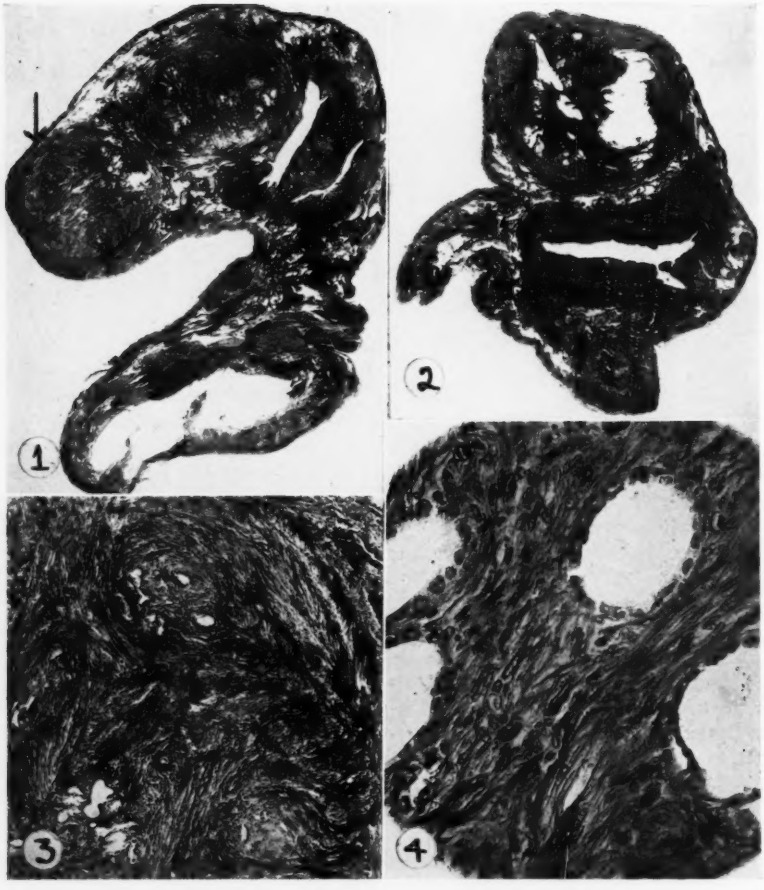

Fig. 1. Cross section of right uterine horn showing lumen. At left are 2 tumors, well encapsulated and separated one from the other. They lie between the longitudinal and circular muscle layers of the uterus. Below is another fibromyomatous growth with a large cystic cavity. Weigert's iron hematoxylin and picrofuchsin. Fig. 2. Cross section of left uterine horn. Above the lumen is an adenofibromyomatous growth with cystic glands and below is another tumor cut near its lower pole. Fig. 3. View at higher power of part of tumor indicated by arrow. The capsule and the glandular islets surrounded by connective and muscular tissue can be seen. Fig. 4. Higher power showing the appearance of glands. Note cuboidal or flattened epithelium surrounded by young connective tissue.

actually produced by the surgical intervention, cannot be stated with certainty. Lipschütz found one pedunculated tumor of a size corresponding to 1 in his scale in the study of many untreated control guinea pigs. This tumor was a subserous fibromyoma similar in structure to those found in women and it did not resemble the experimental fibroids obtained after the injection of estrogen in castrated guinea pigs. We also have failed to find spontaneous uterine tumors in the many untreated control guinea pigs examined.

F. Rodriguez (5) in a study of the doses of estrone necessary to obtain connective tissue tumors in castrated guinea pigs, did not find uterine tumors until after injecting doses over 100 γ or 150 γ three times per week during 3 months. With doses lower than 20 γ he found 1 animal in 37 that presented a small subserous tumor.

The evidence in this case for the experimental production of the tumors by extreme partial castration follows. The apical localization of the growths in the horns is a position shown by Lipschütz and his co-workers to be almost constant when connective tissue tumors appear in the uterus after the injection of estrogens. The size and multiplicity of the tumors is also in accord with what occurs in experimental tumors. The aspect of the tumors is intermediate between that of small spontaneous tumors in guinea pigs and what is obtained by injection of estrogens.

If one considers that these tumors are spontaneous, there still exists the possibility that their size was increased by the operation upon the ovary.

Should it be possible to support this one instance of tumor production as the result of partial castration by other instances, the findings would suggest that there are stages of perturbation of the relationship between hypophysis and ovary, ranging from cystic glandular hyperplasia and atypical proliferation of the endometrium to the rare spontaneous fibromyoma (one case known) and experimental fibromyoma after extreme partial castration (one case). It might be possible to relate these disturbances more closely to the effects produced in the guinea pig by injection of estrogenic hormones, which lead to production of multiple uterine and extragenital tumors.

In this instance, as in the case of cystic glandular hyperplasia produced by extreme partial castration, it is the animal's own hormones that, by their unbalanced state, produce pathological alterations, just as exogenous estrogens administered by injection produce pathologic uterine structures.

SUMMARY

A case is related of an adult guinea pig that, after 14 months of extreme partial castration, showed several apical uterine adenofibromyomas. Although it is possible that these tumors occurred spontaneously, it is probable that they were induced experimentally.

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COMPARATIVE ACTION OF STILBESTROL AND OTHER ESTROGENS ON ENDOMETRIUM OF THE CASTRATED GUINEA PIG[1]

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PROLONGED TREATMENT with follicular hormones induces a glandular cystic hyperplasia of the endometrium in different species including the human being. It would seem probable that the same condition could be produced by artificial estrogens. Two experimental aspects of this question are to be presented in this paper: first, a comparison of the degree of endometrial response to stilbestrol and to natural hormones,[2] and second, peculiarities of the endometrial reaction to stilbestrol.

MATERIAL

Fifty-three castrated female guinea pigs were injected 3 times a week for 6 to 16 weeks with stilbestrol in olive oil. The dosages varied from 0.1 to 1,000γ per injection. Some of the animals in the experiment were part of a series described in a previous paper (1).

Sixty-four castrated guinea pigs were treated for 6 to 16 weeks with dosages of 1 to 400γ estradiol (2, 3). Thirty-five animals were treated with 0.1, 1 and 2γ doses of 4 different esters of estradiol.

Frontal sections of the corpus uteri and transverse section of the horns taken at a level of 1 to 2 cm. above the bifurcation were prepared for microscopic study. They were stained with hematoxylin and eosin and with Van Gieson's stains.

RESULTS

Weight response. Tri weekly injections of 0.1 to 0.5γ stilbestrol for periods of time as long as 3 and 1 months produced, in castrated guinea pigs, uteri which weighed 0.7 to 0.9 gm. Dosages of 1γ for the same period of time produced uterine weights averaging 1.5 gm. This value was somewhat larger than that for uteri in adult virgin females.

Endometrical response. When 1γ stilbestrol per injection was given 3 times a week for more than 3 months, glandular cysts were found in the endometrium of castrated guinea pigs (fig. 1). However, it has been shown (4) that cystic glands and other evidences of endometrial hyperplasia occur spontaneously in normal guinea pigs, so that too much significance should not be attached to the occurrence of cysts such as those present in figure 1.

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[2] The stilbestrol was given by Prof. E. C. Dodds, London, and the natural estrogens by Dr. Karl Miescher of Messrs. Ciba, Basel.

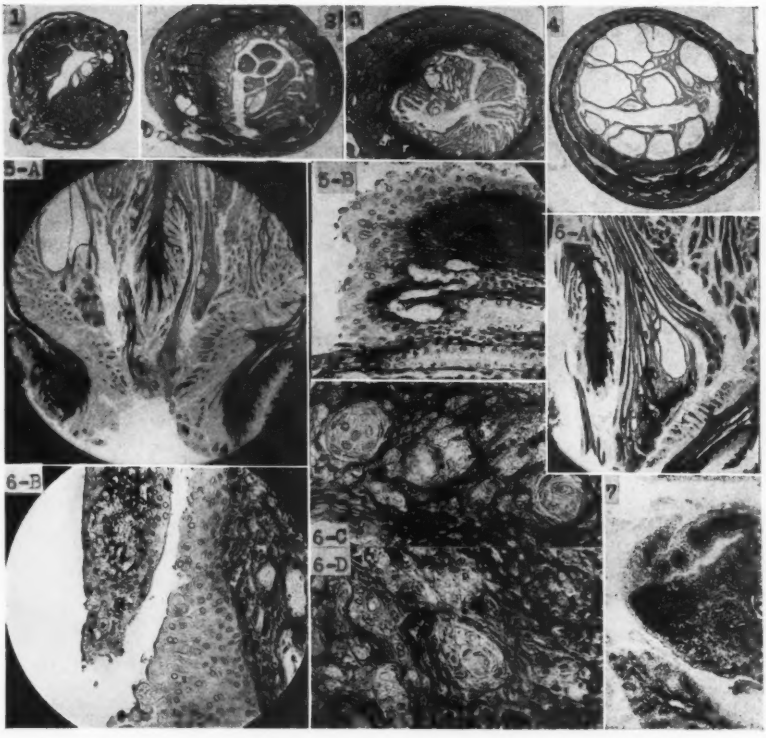


Fig. 1. Uterine horn of castrated guinea pig. 48 injections of 1γ of stilbestrol in the course of 115 days. Normal appearance, though several small glandular cysts are present. ×4.

Fig. 2. Uterine horn of castrated guinea pig. 43 injections of 10γ of stilbestrol in the course of 101 days. Cystic glandular hyperplasia. Uterine cavity crowded with adenomatous polyps. Cystic glands between muscular layers (see two cysts at the right, bottom). ×4.

Fig. 3. Uterine horn of castrated guinea pig. 43 injections of 10γ of stilbestrol in the course of 101 days. Proliferation of endometrium and cystic glands. ×4.

Fig. 4. Uterine horn of castrated guinea pig. 39 injections of 80γ of estradiol in the course of 91 days. Pronounced glandular cystic hyperplasia. ×4.

Fig. 5. Frontal section of corpus uteri of castrated guinea pig. 43 injections of 10γ of stilbestrol in the course of 102 days. *A*. ×4. Septum which separates the individual cervices; portio and fornix to the left and right. Endometrial polyps descending into the cervix. *B*. ×80. Pseudostratification on the distal part of uterine polyp.

Fig. 6. Frontal section of corpus uteri of castrated guinea pig. 39 injections of 80γ of estradiol in the course of 90 days. *A*. ×4. Septum on left; uterine polyp extends to border of portio. The distal part of the polyp is rich in fibrous tissue (small experimental "fibroids" without sharp limits as characteristic of uterine submucosa. *B*. ×100. Pseudostratification of epithelium. *C* and *D*. ×160. "Precancerous" formations in the polyp.

Fig. 7. Frontal section of corpus uteri of castrated guinea pig. 45 injections of 250γ of stilbestrol in the course of 108 days. Distal part of polyp almost reaching vagina. Fibrous transformation of submucosa. ×20.

When dosages of stilbestrol as high as 5γ per injection were given to castrated guinea pigs the number and size of the cysts were greater.

After dosages of 10γ of stilbestrol per injection 3 times a week for less than 6 weeks the endometrial response was greater than that observed after treatment with 5γ stilbestrol per injection. Adenomatous polyps filled the uterine cavity (fig. 2) and in

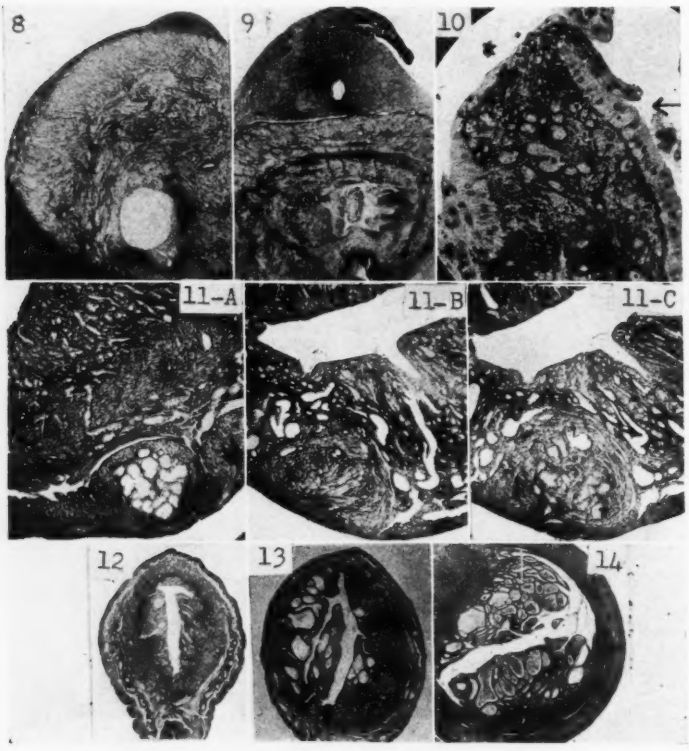


Fig. 8. Uterine horn of castrated guinea pig. 38 injections of 500γ of stilbestrol in the course of 104 days. Endometrium disappeared but some glands still intact. No pyometra. Fibrous submucosa with "fibroids" without sharp limits. Thickened subserosa on the left with neoformation of smooth muscle fibres. Small subserous fibroid on the ventral surface of the horn. ×5.

Fig. 9. Uterine horn of castrated guinea pig. 22 injections of 1,000γ of stilbestrol in the course of 53 days. Mesometrial fibroid involving uterine horn. Pyometra. Endometrium disappeared. Necrotization of the submucosa and of part of myometrium. Leucocytic infiltration between circular and longitudinal layers of myometrium. ×5.

Fig. 10. Uterine horn of castrated guinea pig. 43 injections of 10γ of estradiol in the course of 101 days. Polyp of uterine mucosa. "Angiomatous" submucosa. Endometrium flattened at *. Deformation of cell of endometrium by blood vessel at ←. ×100.

Fig. 11. "Fibroadenomatous" and "fibromyoadenomatous" intramural nodules of the uterine horn of castrated guinea pigs. ×23. A. 46 injections of 5γ of estradiol in the course of 111 days. Tubular structure amidst the myometrium. B. and C. ×23. 39 injections of 3γ of estradiol in the course of 102 days. In B—fibromyomatous periphery of the nodule, in C—adenomatous center.

Fig. 12. Uterine horn of castrated guinea pig. 40 injections of 20γ of estradiol in the course of 96 days. Normal aspect of uterus though several small glandular cysts are present. ×5. To be compared with figure 1.

Fig. 13. Uterine horn of castrated guinea pig. 45 injections of 0.1γ of estradiol given as 17-caprylate in the course of 103 days. Multiple glandular cysts. ×5. To be compared with figure 1.

Fig. 14. Uterine horn of castrated guinea pig. 38 injections of 1γ estradiol given as dipropionate in the course of 85 days. Enormous cystic glandular hyperplasia with formation of polyps. ×5. To be compared with figure 1.

some instances extended into the corpus and through the cervix into the vagina (fig. 5a). The production of this phenomenon has been previously described (5, 6).

A few of these polyps arose from cervical mucosa, but the majority of them developed from the endometrium of the uterine horns. The surface epithelium in the

more distal portion of some of the polyps appeared to have undergone some metaplasia (fig. 5b).

When dosages of stilbestrol as high as 40 to 80γ per injection were used, the characteristics of glandular cystic hyperplasia of the endometrium were more marked (fig. 4). Polyps which descended as low as the vagina frequently occurred (fig. 6a). The epithelium of these polyps was pseudostratified (fig. 6b). Large numbers of epithelial cells concentrically arranged were observed in the stroma of these polyps (fig. 6c, 6d). In some instances there was fibrous tissue present in the subepithelial stroma of the polyps (fig. 7).

When larger dosages of stilbestrol were administered to castrated female guinea pigs, the surface epithelium of the endometrium became necrotic, and the glands and stroma almost completely disappeared (fig. 8). There was an accumulation of pus in the uterine cavity and in the remaining glands and cysts. In some instances there was an infiltration of leucocytes into the submucosa (fig. 9) In other cases a fibrino-purulent peritonitis localized on the uterus and parametrium. Similar conditions produced by the use of estradiol in castrated animals have been previously reported (7).

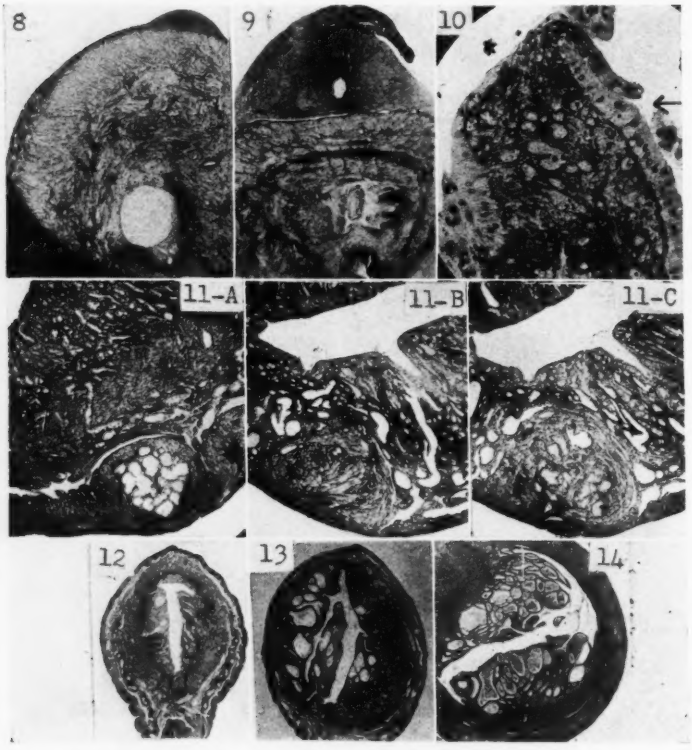
Cystic glandular elements were observed in the myometrium of 2 animals which had received massive amounts of stilbestrol (fig. 11). In one case a structure resembling a minute myofibroadenoma was observed (fig. 11b, 11c).

Comparison of the action of stilbestrol with that of estradiol, free and esterified. The production of the uterine phenomena described above, including necrosis of the endometrium and pyometra have been reported by investigators who have worked with natural estrogens in mice (8, 9). The action of estrone, of estradiol and its esters 3-benzoate, 3-17-propionate, 17-benzoate-3-n-butyrate and 17-caprylate on the uterus of the castrated guinea pig was investigated. It is sufficient to compare the uterine response in animals injected 3 times a week with 10γ stilbestrol per injection for 3 months (fig. 2, 3) with the response in animals injected with 10 to 20γ of free estradiol for the same period of time (fig. 12). As described in the section above, there was a marked degree of glandular cystic hyperplasia and adenomatous polyps which descended into the vagina in castrated guinea pigs receiving stilbestrol (fig. 5a). After treatment with 20γ of estradiol per injection for the same period of time, there was no evidence of glandular cystic hyperplasia. The condition of the endometrium after this dosage of estradiol for 3 months was similar to that observed after castrated animals had received 1γ of stilbestrol for 3 months.

A comparison of the uterine response to stilbestrol with that to estradiol is valid only when the intervals of treatment are the same with the 2 substances, since glandular cystic hyperplasia may be obtained with smaller quantities of free follicular hormones when these are injected daily (10). Subcutaneous pellets of estradiol or of estriol can produce a cystic glandular hyperplasia in periods of time as short as 7 to 20 days (11, 12).

When 1γ of monobenzoate or dipropionate of estradiol per injection was given for 16 weeks (fig. 14) adenomatous polyps filled the uterine cavity. Glandular cystic hyperplasia was produced with as small a dose as 0.1γ of the monocaprylate (fig. 13). Figure 1 is a section from the uterus of an animal receiving 1γ stilbestrol per injection.

Vascular response of the endometrium to stilbestrol. There is an increased frequency of uterine bleeding associated with the use of stilbestrol as compared with the use of follicular hormones (1). Bleeding may occur in animals receiving as little as 5γ stilbestrol per injection. With larger dosages of stilbestrol the endometrial stroma of a polyp often consisted largely of capillaries. The polyp was so distended that the epithelium was deformed and flattened (fig. 10). An increased vascularity, especially in polyps was observed after treatment with natural and esterified estradiol, but the



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increase was not as remarkable as that occurring when stilbestrol had been used.

Other effects of stilbestrol. A decreased growth rate in rats treated with stilbestrol has been reported (13). The initial weights of the guinea pigs employed in these experiments were so varied that it can only be said that the increase in weight was very slow in animals which received massive dosages of stilbestrol.

There was a loss of hair, especially on the abdomen in several guinea pigs, as has been reported in experiments in which follicular hormones have been given for prolonged periods of time (14, 15) and in experiments in which long follicular phases are experimentally established by ovarian transplantation of fragmentation (4).

DISCUSSION

That quantity of estrogen in an oily medium which, when given 3 times a week to a castrated guinea pig will maintain the normal uterine weight may be designated as a hysterotrophic dose of estrogen.

The weight of the uterus of a castrated guinea pig under the influence of prolonged treatment with estrogens depends upon the age and weight of the animal at the time of castration. In general, the weight of such uteri is greater in animals which had borne young or which were sexually mature at the time of castration.

These factors were not taken into consideration in attempting to determine a useful hysterotrophic dose of the various estrogens.

If a minimal quantity of an estrogen, for example 0.1γ of monocaprylated estradiol is injected 3 times a week for 3 months, the uterus may be maintained at a normal or sub-normal weight but an atypical response of the endometrium, such as excessive proliferation, may occur. It becomes apparent, then, that this hysterotrophic dose is arbitrary and not physiologic.

However, the determination of hysterotrophic dosages of some of the estrogens is of value for comparative work on the toxic and tumorigenic effects of stilbestrol and estradiol.

The hysterotrophic dose of estradiol in castrated guinea pigs is 1γ per injection 3 times a week for 3 months. A dose as high as 20γ per injection of estradiol 3 times a week for the same period of time fails to produce glandular cystic hyperplasia (1). The ratio between the toxic uterine dose and the useful hysterotrophic dose is greater than 20.

The useful hysterotrophic dose of stilbestrol is somewhat larger than 0.5γ per injection 3 times a week for 3 months. When 5γ per injection is given, glandular cystic hyperplasia begins to be conspicuous and is accentuated with 10γ per injection. The ratio between the toxic uterine dose and the useful hysterotrophic dose of stilbestrol is probably about 10.

The ratio between toxic and hysterotrophic doses of the benzoic and dipropionic esters of estradiol is smaller than 10, the toxic dose being less than 1γ (fig. 14), the useful hysterotrophic dose more than 1γ.

The ratio between toxic and hysterotrophic doses of stilbestrol is intermediate between that of the free hormones and of the esters of estradiol.

It has been suggested (1) that the guinea pig may lack the capacity to inactivate the artificial estrogens with the same facility that it does the natural follicular hormones. This suggestion was based on certain observations referring to the comparative increase of uterine weight under the influence of estradiol and stilbestrol. Stroud (16, 17) reported that 20 to 25% of the stilbestrol and hexestrol was recovered from the urine of the rabbit. Injected estradiol, on the other hand, is converted to estrone and estriol (18, 19). A higher level of estrogenic material can be maintained with stilbestrol which is resistant to conversion and inactivation than can be main-

tained with estradiol. When stilbestrol is administered subcutaneously its action resembles that of esterified estradiol (20). Hexestrol produced results similar to those obtained with stilbestrol and esterified estradiol (21).

SUMMARY

Prolonged treatment of castrated female guinea pigs with stilbestrol produced glandular cystic hyperplasia of the endometrium, excessive pseudstratification of the epithelium, adenomatous polyps which descended into the vagina, epithelial masses in the stromal tissue which resembled epithelial pearls, fibrosis of some stromal areas, pyometra, necrosis of the endometrium and of the uterine wall and fibrinous purulent peritonitis.

When the spacing of the injections of stilbestrol and of free follicular hormones was the same, toxic reactions appeared with smaller doses of stilbestrol than with the natural estrogens. The useful hysterotrophic dose of stilbestrol likewise was smaller than that of the free follicular hormones.

The difference between the effectiveness of stilbestrol and the natural hormones is tentatively explained on the basis of the greater resistance of the artificial estrogens to inactivation.

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THE INHIBITION OF COPPER-INDUCED OVULATION IN THE RABBIT BY PROGESTERONE

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IT IS POSSIBLE TO PRODUCE OVULATION in the rabbit either by provoking a discharge of the pituitary gland, for example, by permitting copulation, or by injecting some gonadotropic agent which can act directly on the ovary. The discovery that copper salts can provoke ovulation in the rabbit has presented the problem of the mechanism of this interesting reaction. Fevold, Hisaw, and Greep (1) originally advanced the hypothesis that the action of copper was due to the augmentation of the action of subthreshold quantities of some hormone normally present in the rabbit blood. Recent experiments (2) however, afford strong evidence that copper-induced ovulation in the rabbit is due to a pituitary discharge which is brought about by impulses carried to the gland by way of the stalk.

The present experiments with progesterone were initiated several years ago, and were originally planned to analyze and compare the gonadotropic action of plant extracts and copper salts. At that time the best gonadotropic preparations from plants were so crude that it was necessary to give some consideration to the small amounts of copper present in green leaves. Since that time progress with the study of plant gonadotropins has been so satisfactory (3) that the experiments with progesterone are no longer so important from that standpoint. Nevertheless, they furnish further evidence for the site of action of copper in the rabbit and emphasize the value of progesterone for the analysis of the mechanisms of ovulation.

METHODS AND MATERIALS

Except for the group of isolated females listed in table 1 and the group of pseudopregnant animals listed in table 3, all of the rabbits used in these experiments were healthy postpartum animals selected to meet the rigid assay standards previously described (4).

The progesterone[2] was a crystalline preparation dissolved in sesame oil. It was obtained from the same source as the progesterone used in our earlier experiments (5). The progesterone was administered subcutaneously and except for one group of animals (table 2, *exp.* 1) was given in several doses of 2 mg. each, over a period of 24 to 72 hours.

The copper salts were C. P. copper acetate and copper sulphate, made up in 1.0 and 1.25% solutions respectively, so that 1 cc. of either solution contained approximately 3.2 mg. of copper ion. The provocative dose of copper was given in a single intravenous injection. In those experiments where progesterone had been adminis-

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[2] We are indebted to Dr. Erwin Schwenk of Schering Corp., Bloomfield, N. J., for the progesterone used in these experiments.

tered before hand the provocative dose of copper was injected 4 to 5 hours after the final dose of progesterone.

In some previous unpublished experiments with copper salts we had determined that the latent period of the ovulatory reaction was less than 18 hours in postpartum animals. Consequently, in the present experiments the exploratory laparotomy was performed 18 to 24 hours after the copper injection. The nature of the ovarian response (presence or absence of fresh rupture points) was determined by gross inspection.

RESULTS

From the data of table 1 it may be seen that a dose of 3.2 mg. of copper ion was sufficient to provoke ovulation in most of the isolated rabbits[3] and was definitely above the threshold for the reaction in the postpartum female. The limited number of observations does not permit a satisfactory location of the level of the 50% response. If the distribution of positive and negative responses in these samples is representative of the true levels of response, the unit dose of copper for this reaction (50% response) would be less than 2.4 and greater than 1.6 mg. per rabbit.[4]

TABLE 1. RESPONSE OF THE FEMALE RABBIT TO INTRAVENOUS INJECTIONS OF COPPER SALTS

Type of animal	Anion	Cu ion	Ovulation	
			+	−
		mg.		
isolated	sulphate	3.2	7	2
post partum	acetate	3.2	6	0
post partum	acetate	2.4	4	1
post partum	acetate	1.6	0	7

TABLE 2. RESPONSE OF THE POST-PARTUM RABBIT TO COPPER SALTS AFTER TREATMENT WITH PROGESTERONE

Experiment	Progesterone		Copper ion	Ovulation		P[2]
	Total dose	Injection period		+	−	
	mg.	*hrs.*[1]	*mg.*			
1	3	48	3.2	2	2	0.08
2	6	24	3.2	1	3	0.007
3	8	44	3.2	0	1	—
4	6	48	4.0	1	7	0.0001

[1] Total elapsed time between the first and last injections of progesterone.

[2] P = probability of encountering this distribution by chance, assuming that the injection of 3.2 mg. or more of copper ion intravenously will produce ovulation in 85% of untreated post-partum rabbits.

When the provocative dose of copper was administered to animals which had been treated with progesterone the reaction was definitely inhibited (table 2). The first few experiments with progesterone were for the purpose of finding an injection schedule which would prove effective and yet require a minimum of progesterone (table 2, *experiments* 1, 2 and 3). It is questionable if a total of 3 mg. of progesterone

[3] These isolated rabbits were in good health, except for slight cases of snuffles. They had not been pregnant recently, and were comparable to the females so frequently, and erroneously, designated as "estrous" in many present day publications.

[4] The rabbits used for these experiments weighed between 3.6 and 4.0 kg. No effort was made to correlate the response with the body weight.

(1 mg. daily for 3 days) produced any inhibition of the copper response. If we assume that 3.2 mg. or more of copper ion will produce ovulation in more than 85% of postpartum rabbits, a distribution like that in *experiment* 1 should be encountered by chance about once in every 12 samples of 4 ($P=0.08$). With the higher doses of progesterone, however, the inhibition is unquestionable.

Concurrently with *experiment* 4 of table 2 we tested the reaction of progesterone-treated animals to a plant extract. This particular extract was a crude one made from oat plant juice. A dose of 125 mg. was required to produce ovulation in untreated postpartum rabbits. For this experiment 5 rabbits were given a total of 8 mg. of progesterone over a period of 3 days (2 mg. once daily). Four hours after the last injection of progesterone, each of the 5 rabbits was given an injection of a solution representing 200 mg. of the oat extract (about 1.6 times the minimal effective dose). Despite the relatively high dose of progesterone the response to the plant extract was not inhibited since each of the 5 animals ovulated. If the active agent in the plant extract had been copper or some similarly acting agent we should expect a series of 5 positive responses like this less than once in a thousand times if *experiment* 4 of table 2 is accepted as being representative.

TABLE 3. RESPONSE OF PSEUDOPREGNANT RABBITS TO INTRAVENOUS INJECTION OF COPPER SALTS

The provocative dose of copper was 4.8 mg. administered as the acetate in one intravenous dose. The stage of pseudopregnancy indicated in the table refers to the day on which the copper salt was injected.

Stage of pseudopregnancy	Ovulatory response to copper	
	+	−
days		
18 to 19	4	0
16	2	1
15	0	2
13	0	3
10	1	1

For comparative purposes we tested the copper reaction in a few pseudopregnant animals, that is, animals under the influence of the progesterone secreted by their own corpora lutea (table 3). The positive reactions toward the end of pseudopregnancy were anticipated. From our results with the progesterone injections we had also anticipated the inhibition of the copper reaction at the 13th to 15th days of pseudopregnancy. The one positive response to copper on the 10th day of pseudopregnancy is a noteworthy inconsistency.

DISCUSSION

When these experiments were first designed there may have been some reason to question the nature of the gonadotropic extracts prepared from green leaves. The chemical properties of the active material, particularly its instability at low temperatures, were evidence against the possibility of any simple inorganic substance being the active material. Nevertheless, the early plant extracts were very crude. In many instances more than 300 mg. were required to produce ovulation in the rabbit and this dose often represented several hundred gm. of the original dried plant. The copper content of green plants has been reported to be from 4 to 14 mg. per kg. of dried plant (6). In oat plants a concentration of 10 mg. per kg. was found in a sample grown on soil fertilized with copper salts (6). Since the threshold of the ovulatory response to copper is about 2.4 mg. it was conceivable that biologically significant amounts of copper, perhaps in some combined form, could be carried through the extraction process into the final preparation.

More recent experiments with extracts prepared from the fresh juice of green leaves (3) have largely eliminated the necessity of considering such possibility. The extracts prepared from canned juice of the oat plant produced ovulation in doses equivalent to 20 to 50 cc. of the fresh juice. For our best preparation from the immature corn plant the ovulatory dose was less than 9 mg. of the final product, and this represented about 3 cc. of the fresh juice. Since these plant juices are about 90% water the copper content should be of the order of 1 mg. per liter. Hence a dose of extract representing 1,000 cc. of fresh juice would not contain enough copper to produce ovulation even if the entire copper content were carried quantitatively into the final product.

Some preliminary experiments have shown that our plant extracts are capable of stimulating the ovaries of normal, immature rats. With total doses of 2 to 3 times the ovulation dose in the rabbit the rat ovaries showed an increase in weight of about 100% and exhibited discrete corpora lutea. Since copper does not stimulate the immature rat ovary (7) we must attribute the gonadotropic activity of the plant extracts to something other than copper.

The present experiments with progesterone emphasize the difference between the action of copper and the action of the plant gonadotropin. The progesterone prevented the response to copper but was unable to prevent the ovulatory response to the oat extract. In earlier experiments (8) we presented evidence that progesterone inhibits postcoital ovulation by blocking the discharge of gonadotropic hormone(s) from the pituitary gland. Surgical section of the pituitary stalk accomplishes the same thing (9). To this extent, animals with complete stalk section are physiologically comparable to estrous rabbits injected with adequate doses of progesterone. In both types of animal the ovarian follicles retain their normal sensitivity to peripheral gonadotropic stimulation (5, 9), and yet fail to ovulate in response to coitus or to the intravenous injection of the copper ion.

Neither the rabbit with complete stalk section nor the animal treated with progesterone is the equivalent of a completely hypophysectomized rabbit. In the latter corpora lutea degenerate unless special treatment for their maintenance is instituted. In the stalk-sectioned animal once corpora lutea have been formed by a single gonadotropic injection, they grow normally and reach full functional development (10). Obviously progesterone does not completely block all pituitary secretion for the rapid growth and development of the corpora lutea during early pseudopregnancy normally occurs in the presence of effective concentrations of progesterone. We must distinguish, therefore, between the complete absence of pituitary secretion and the type of pituitary block produced either by stalk section or by progesterone injections. The pituitary block obtained by these procedures merely prevents the explosive release of gonadotropic hormone(s) which is necessary to rupture the ripened follicles and to stimulate the early growth of the corpora lutea (11). This type of block does not prevent the levels of pituitary secretion needed for follicular maturation or for the maintenance of previously formed corpora lutea.

Unfortunately, the progesterone treatment of estrous rabbits is not quite as effective as complete stalk section for inhibiting ovulation. With the doses of progesterone employed in our experiments an absolute inhibition of the ovulatory response to copper was not attained (cf. *exp.* 4, table 2). Even the physiologic level of progesterone prevailing in pseudopregnant rabbits does not always prevent the response to copper. This is shown not only by the one positive response on the 10th day of pseudopregnancy in our own series, but also in a similar example in the series of animals recently reported by Emmens (12).

The fact that the progesterone inhibition is not always complete makes it impos-

sible to accept the progesterone block as a completely satisfactory substitute for pituitary stalk section. The inhibitory actions of atropine and ergotamine are also somewhat short of ideal substitutes for surgical procedures, but this does not detract from their value as implements for physiological and pharmacological studies. To a similar degree it seems justifiable to expect that progesterone will prove useful in the analysis of the ovulatory response of the rabbit, differentiating between substances which act peripherally on the ovary and those which act only by excitation and discharge of the pituitary.

SUMMARY

In the postpartum rabbit a single intravenous injection of a copper salt produces ovulation in less than 18 hours. The ovulatory response is regularly obtained with 3.2 mg. of copper ion. It is estimated that the level of 50% response to the injection of copper is at a dose less than 2.4 mg. and more than 1.6 mg. of copper ion.

It is possible to inhibit the ovulatory response to copper by adequate doses of progesterone administered for several days previously. Such progesterone treatment did not inhibit the ovarian response to a gonadotropic extract prepared from the juice of young oat plants.

Although the pituitary inhibition produced by progesterone is not quite complete, the available evidence indicates that progesterone-treated postpartum rabbits are physiologically comparable to rabbits in which the pituitary stalk has been severed.

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A QUANTITATIVE STUDY OF THE INHIBITION OF THE OVARY AND OF THE TURGESCENT PERINEUM OF THE NORMAL BABOON PRODUCED BY A SINGLE INJECTION OF ESTRADIOL BENZOATE[1]

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THE OBJECT OF THIS PAPER is to record the effects of single injections of varying amounts of estradiol on the behavior of the perineal (sexual) swelling and on the length of the menstrual cycle in the baboon (*Papio porcarius*) when given on the 8th to 10th day of the menstrual cycle.

An assessment is made of the action of exogenous estrogen on the ovary as determined by the reaction of the perineal swelling and by the alteration in the length of the menstrual cycle. At the same time the possibility is considered of determining by an indirect approach, the amount of estrogen elaborated by the ovary on a particular day in the first part of the cycle.

At the same time the mechanism whereby menstruation is postponed by the administration of estrogen is discussed in the light of the facts presented in this study. Attention is also drawn to the fact that whenever estrogen is used therapeutically in sufficient amounts, among its various effects it will also produce ovarian inhibition.

MATERIAL AND METHODS

The baboons used were normal uncastrated mature animals whose cycles had been observed for at least 6 months. Except for the unerupted last molars in two instances all the baboons were in possession of their complete permanent dentition.

The daily fluctuations in the size of the perineum were followed in the manner already described (3, 4). Injections of estradiol in sesame oil[2] were given intramuscularly.

EXPERIMENTAL

Experiment 1. (*Baboons 1–4*, table 1). The first series of experiments was planned to discover if estradiol benzoate given as a single administration in the first part, or turgescent phase of the cycle, could interfere in any way with the normal course of the cycle as assessed by the behavior of the perineum.

Of the 4 baboons, one normally showed a slow initial rise of the perineum after menstruation. This was not considered a disadvantage. All the animals received 5 mg. of estradiol benzoate in one dose on the 8th to 10th day of the cycle. The perineal response of one is graphed in figure 1.

Following the injection, the perineum continued to swell for 4 to 6 days in the case of *baboons 1*, 3, 4, and for 11 days in *baboon 2*, whose perineum normally rose

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very slowly after menstruation. After this initial increase in size the perineum deturgesced and 10 to 12 days after the injection it returned to the resting state such as is usually seen in normal animals a few days before menstruation. The resting state was maintained for 3 to 6 days and then without the intervention of bleeding perineal turgescence was initiated and the sequence of events thenceforth was not unlike that encountered in a normal cycle (fig. 1). On the 53rd to 60th day of the cycle, or 40 to 50 days after the administration of estrogen, bleeding occurred when the perineum had once more achieved its resting level (table 1). In all 3 baboons the cycles were lengthened by 15 to 20 days, and all 3 animals responded broadly in the same fashion.

This experiment demonstrated that a single administration of 5 mg. of estradiol in the first part of the cycle not only inhibited perineal turgescence of the baboons but actually caused a complete deturgescence of the perineum, and thereafter the perineal rhythm was reestablished but the cycle was prolonged by 15 to 20 days.

Experiment 2. (*Baboons* 5–8, table 1). The object of this experiment was to dis-

TABLE 1. EFFECT ON THE PERINEAL SWELLING AND ON THE LENGTH OF THE MENSTRUAL CYCLE AFTER A SINGLE INJECTION OF ESTRADIOL BENZOATE ON THE 8TH TO 10TH DAY OF THE CYCLE IN BABOONS

Baboon No.	Estradiol	Earliest effect on perineum after injection	Length of effect of estradiol on perineum	Length of normal cycle	Length of experimental cycle
	mg.	*days*	*days*	*days*	*days*
1	5.0	6	18	42	60
2	5.0	11	15	40	60
3	5.0	4	15	37	53
4	5.0	5	—	38	—
5	1.0	6	15	40	60
6	0.1	4	10	38	44
7	0.1	4	10	38	45
8	0.1	5	16	38	26
9	0.05	nil	nil	38	38
10	0.05	nil	nil	39	38

cover if amounts of estradiol less than 5 mg. could produce the same perineal reactions as obtained with 5 mg. estradiol benzoate. Accordingly *baboon* 5 received 1 mg., *baboons* 6, 7 and 8 received 0.1 mg. and *baboons* 9 and 10 each received 0.05 mg. of estradiol benzoate. From previous work (2) it was shown that a total of 0.05 to 0.2 mg. of estradiol given over 10 days is the minimum total amount which will cause estrogen-withdrawal bleeding in castrated or immature baboons. *Baboon* 5, which received 1 mg. estradiol benzoate responded in a manner similar to that described in *experiment 1* (table 1). The cycle was prolonged by 20 days.

After receiving 0.1 mg. of estradiol the perineum of *baboon* 8 continued to swell rapidly until the 13th day of the cycle and thereafter underwent deturgescence reaching its resting level on the 23rd day; bleeding unexpectedly occurred on the 26th day, or 16 days after the injection (fig. 2). The cycle was shortened by 12 days.

Baboons 6 and 7 reacted in a manner similar to one another but different from *baboons* 1 to 5 (fig. 2). Forty-eight hours after the injection of 0.1 mg. estradiol maximum turgescence was attained. Within the next 24 hours there was a slight decrease in size of ½ to 1 inch. On the 16th day, or 6 days after the injection, the perineum had decreased in size by 2 inches (*baboon* 6) and 2½ inches (*baboon* 7). At this time the perineum was 2½–3 inches above the resting level. On the 20th day of the cycle (10 days after the injection) the perineum commenced to swell once more and continued, until the 24th to 26th day, and then returned to its resting level (fig. 2). Bleeding occurred on the 44th to 45th day. The cycle in each of these two baboons

was prolonged by 6 and 7 days respectively (table 1). *Baboons* 9 and 10 did not show any reaction to injections of 0.05 mg. of estradiol benzoate.

Thus in *baboons* 6 and 7, 0.1 mg. of estradiol caused a temporary but partial deturgescence of the turgescent perineum and the cycle was prolonged, whereas in *baboon* 8 the perineum underwent deturgescence and bleeding occurred prematurely and the cycle was shortened. In *baboons* 9 and 10, the amount of estradiol was inadequate to cause any recognizable change in the menstrual cycle.

DISCUSSION

It has been shown in experiments on castrate or immature baboons that perineal turgescence is caused by estrogen (3, 4), whereas perineal deturgescence in such ani-

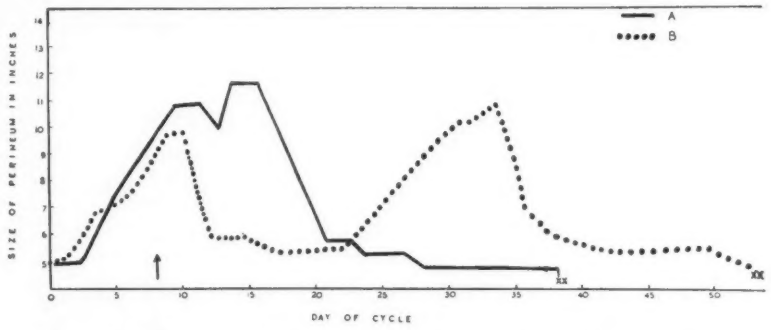

Fig. 1. A, Normal cycle. B, *Baboon* 3 injected on the 8th day of cycle with 5 mg. estradiol benzoate. X, Onset of bleeding. Arrow indicates day of injection.

mals is due to withdrawal of estrogen or it can be induced by progesterone in the presence of enormous quantities of estrogen (5). In normal, ovulating baboons perineal deturgescence is caused by endogenous progesterone or it can be artificially produced by giving progesterone in the first part of the cycle (6, 7).

In *experiment* 1, in which 5 mg. of estradiol evoked complete perineal deturgescence, the question naturally arose as to how this reaction was accomplished. On the basis of previous investigations mentioned above there were two possibilities, namely, that exogenous estrogen excited the formation of a corpus luteum prematurely, or else the exogenous estrogen in some way led to a temporary inhibition of the ovaries with a consequent suppression of estrogen. The answer was obtained by removal and histological examination of the ovaries of *baboon* 4. No recently formed corpus luteum was discovered and the graafian follicles were damaged. It was, however, desirable for future experimental work to discover whether this information could be obtained by a study of the behavior of the perineal swelling alone.

On comparing the effects of a single injection of progesterone and of estrogen given in the first part of the cycle to mature animals there are striking but clear-cut differences in the behavior of the perineum. Estrogen first causes the perineum to turgesce for 4 to 6 days (*baboons* 1, 3, 4) or 11 days (*baboon* 2), and thereafter the perineum undergoes deturgescence gradually, to reach a resting level (fig. 1). At the end of 16 days after the administration of estrogen and without the onset of bleeding the perineum goes through another almost normal but slightly abbreviated cycle. The experimental cycle is prolonged by 15 to 20 days. Progesterone (5–15 mg.) however, causes the perineum to undergo rapid deturgescence and the resting level is attained

by the 4th to 7th day and then, without the intervention of bleeding, the perineum also passed through what must be regarded as a new and almost normal but abbreviated cycle. The experimental cycle is lengthened usually by 5 to 8 days. The immediate effect of progesterone wears off in from 4 to 7 days, although of course, the cycle is prolonged, but usually not to the same extent as after estrogen. If perineal turgescence is induced in castrate or immature baboons by estrogen and if the estrogen is withdrawn then the perineum remains at its high level or it may even continue to rise for 2 to 3 days, but 10 to 16 days after the withdrawal of estrogen the perineum is at rest and bleeding occurs. The whole sequence of events after the withdrawal

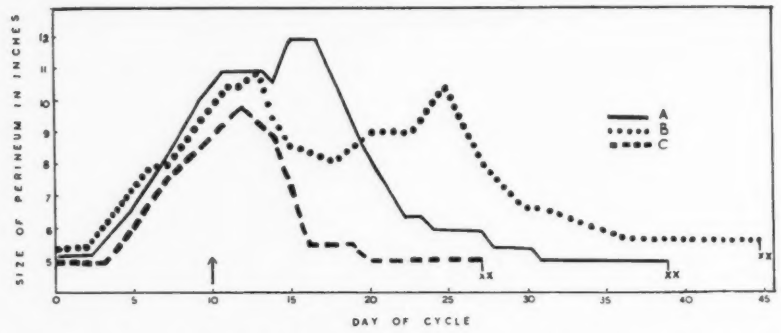

Fig. 2. A, Normal cycle. *B*, *Baboon* 7 injected with 0.1 mg. estradiol benzoate on the 10th day of cycle. *C*, *Baboon* 8 injected with 0.1 mg. estradiol benzoate on 10th day of cycle. X, Onset of bleeding. Arrow indicates day of injection which was the same in both baboons.

of estrogen in castrates is very similar to that produced by 1 to 5 mg. of estradiol in mature baboons given in the first part of the cycle, except that in the latter, after perineal rest had been induced, bleeding does not supervene and the perineum commences to swell once more.

The problem of assessing the nature of ovarian activity by an examination of the perineum is slightly complicated by the fact that in *baboon 8* after perineal rest had been achieved, bleeding occurred 16 days after the giving of the estrogen. Where sufficient progesterone is given in the first part of the cycle bleeding also occurs, but in this case within 4 to 7 days. Besides, the perineal deturgescence which is induced after progesterone is much more precipitous than it is after estrogen. In the case of *baboons* 6 and 7 partial deturgescence was produced, which so far has not been noticed after giving small quantities of progesterone. In this respect progesterone acts according to the 'all or none law.'

The evidence goes to show that, from a careful study of the behavior of the perineum in the baboon, very accurate information can be obtained as to how the ovaries are reacting to exogenous estrogen. This eliminates the inconvenient number of laparotomies and ovariectomies which would be unavoidable in investigations of this nature. From the study of the perineum it follows that estrogen given as a single injection in the first part of the cycle inhibits the production of estrogen by the ovary which in turn causes perineal deturgescence and perineal rest. The ovary recovers within 10 to 16 days and estrogen is once more elaborated and the perineum commences to turgesce. This inhibition of the ovary was associated with estrogen withdrawal bleeding in one baboon only (*baboon 8*).

It has been established that estrogen given repeatedly in the first part of the cycle

can postpone menstruation in women (11). In fact Zondek has delayed menstruation in this way for as long as 70 days. Zondek was of the opinion that estrogen inhibits the ovary by interfering with the production of prolan A (10). Whether this is the explanation or not, does not affect the main issue raised here, namely, that the ovaries bear the brunt of the burden. By giving estrogen continuously to baboons the perineum is maintained in a state of maximum turgescence and when the estrogen is withdrawn the perineum undergoes deturgescence. It must be appreciated that the postponement of menstruation by estrogen is due to the inhibition of the ovaries. This may be of a temporary nature, as in the case of the *Macacus rhesus* after repeated small quantities (1) or in the baboon after a single administration of estrogen. When estrogen is administered over long periods the ovaries are completely inhibited and the length of time that menstruation is postponed depends entirely upon the maintenance of the exogenous estrogen. As soon as exogenous estrogen is suspended, then, after a definite latent period, estrogen withdrawal bleeding is precipitated.

It can be stated almost categorically therefore, that, provided it is given in the first part of the cycle, estrogen in sufficient quantities will cause ovarian inhibition within 4 to 11 days, the duration and extent of this inhibition is dependent almost exclusively on the time over which adequate amounts of estrogen are repeatedly administered. In the baboon a single injection of 1 to 5 mg. causes inhibition for 10 to 16 days; in the case of the *Macacus rhesus*, judging from figure 1 of Ball and Hartman (1), repeated injections of small quantities of estrogen inhibited the ovaries for 8 to 10 days; after a single injection of 5 mg. of estrogen on the 8th day of the cycle the ovaries in women are also inhibited for 8 to 10 days and the cycle may be shortened by 8 to 10 days or lengthened by as much as 16 to 18 days (8).

It is known from studies of other endocrine glands that the action of a gland can be inhibited by administering the essential factors secreted by that gland. This has been shown for the parathyroid (9). It seems from the data presented above that the ovary is no exception to this rule.

Since in the baboon the administration of exogenous estrogen leads to measurable changes in the perineum and of the length of the cycle and that these changes are due to ovarian inhibition, it seems possible to assess the daily output of estrogen by the ovaries by an indirect manner. This is evident from the following considerations. The perineum of the baboon responds in two ways to estrogen, namely, by undergoing partial deturgescence after 0.1 mg. of estradiol (*baboons* 6 and 7, table 1) and complete deturgescence after 1 to 5 mg. of estradiol. Since the deturgescence is produced by inhibiting the ovary and thus by suppressing the amount of estrogen elaborated by that organ it might be reasonable to presume that the ovaries were completely inhibited by 1 to 5 mg. of estradiol and therefore the ovaries were elaborating less than the equivalent of 1 to 5 mg. of estradiol benzoate. Similarly 0.1 mg. of estradiol benzoate caused partial deturgescence in two baboons and complete deturgescence in one and it seems possible that the ovaries might be producing less than or almost the equivalent of 0.1 mg. of estradiol. In two baboons the perineum was unaffected by 0.05 mg. of estradiol. From this it would appear, provided the premises are correct, that the ovary of the baboon is producing the equivalent of or slightly less than 0.1 mg. of estradiol benzoate on the 8th to 10th day of the cycle.

As already mentioned, it is not possible to follow as easily the day to day reactions of the woman as of the baboon, but in the human female the cycle may also be lengthened or shortened by estradiol benzoate administered in the first part of the cycle. It is not unreasonable to suppose that the modus operandi of estradiol in the human female in this respect is not unlike that in the baboon. By determining the minimum amount of estradiol benzoate necessary either to lengthen or shorten

the cycle it might be possible also to discover the quantity of estrogen elaborated by the ovary, at least in the first part of the cycle.

SUMMARY AND CONCLUSIONS

The sexual swelling of the baboon has once more proved to be a valuable indicator in experiments involving the use of the ovarian hormones.

It has been demonstrated that a single injection of estradiol benzoate given to mature baboons in the first part of the cycle upsets the normal behavior of the perineum and, in all baboons, with one exception, the cycle is lengthened.

The behavior of the perineum depends almost exclusively on the amount of estradiol used. One to 5 mg. of estradiol caused a temporary rise of the perineum (3–4 days) and then a slow deturgescence and finally perineal rest 10 to 15 days after the injection. Thereafter, without the intervention of bleeding, a new but slightly abbreviated and almost normal perineal rhythm was initiated. The experimental cycle was lengthened. In 2 of 3 baboons 0.1 mg. of estradiol benzoate caused a partial temporary deturgescence of the perineum and then the cycle continued normally thereafter, although it was lengthened, but not to the same extent as after 5 mg. of estradiol. In one baboon perineal deturgescence was complete and bleeding occurred 16 days after the injection, and the cycle was shortened. In two baboons 0.05 mg. of estradiol produced no visible alteration in the perineal rhythm or in the length of the menstrual cycle.

Evidence has been brought forward to show that a single injection of a sufficient amount of estradiol benzoate inhibits the ovary of the baboon for 10 to 16 days.

The postponement of menstruation by a repeated administration of estrogen is produced by ovarian inhibition and the length of the postponement is dependent on the maintenance of the supply of exogenous estrogen. On withdrawal of the estrogen, bleeding is precipitated after the usual latent period.

The minimum amount of exogenous estrogen necessary to inhibit ovarian activity in the baboon on the 8th to 10th day of the cycle was estimated as being approximately 0.1 mg. or less of estradiol benzoate. It was suggested that this technic of inhibiting the ovary might be employed for determining the amount of estrogen secreted by the ovary on any particular day in the first part of the cycle in women as in baboons.

It is a pleasure to express my indebtedness to Professor R. A. Dart for his advice and encouragement. My thanks are due to Miss C. Gilbert and Mr. Dry for assistance with the daily measurements of the baboons.

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ACTION OF PROGESTERONE ON THE GENITAL ORGANS OF THE UNPRIMED RHESUS MONKEY

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THE CORPUS LUTEUM HAS LONG BEEN RECOGNIZED as the organ responsible for many of the specific structural and functional modifications which occur in the secondary sex organs of mammals during the reproductive period. Among these changes may be mentioned the progestational proliferation of the endometrium and the lobular development of the mammary gland. In the menstruating primates the corpus luteum also plays an important although less clearly defined rôle in the initiation of menstruation. In the course of physiologic events the corpus luteum exercises its functional activity upon organs which have previously been subjected to estrogenic stimulation; and in the primates at least, the luteal body continues to produce estrogenic hormone in addition to its specific secretion, progesterone.

Previous experiments designed to test the various functions of progesterone have indicated the necessity for the preliminary or concurrent administration of estrogens (the "one-two reaction" of Hisaw). Castrated animals, when treated with progesterone alone, have with few exceptions proved unresponsive. The observations herein reported show that crystalline progesterone in adequate dosage is capable of inducing some of its specific effects in the castrated monkey without preliminary priming with estrogens.

EXPERIMENTAL

Four experiments were performed in which 3 mature female rhesus monkeys were used which had been surgically castrated 37 days to 4 years previously. The animals were injected subcutaneously with 5 to 20 mg. daily of crystalline progesterone as follows.

No. 623[1] (castrated 50 dy.)	5 mg./dy.,	5/9/39 to 6/10/39 (32 dy.)
No. 673[1] (castrated 37 dy.)	20 mg./dy.,	5/9/39 to 6/10/39 (32 dy.)
No. 673[1] (castrated 8½ mo.)	10 mg./dy.,	12/17/40 to 1/4/41 (19 dy.)
No. 145C[2] (castrated 4 yrs.)	20 mg./dy.,	1/31/41 to 2/26/41 (27 dy.)

Biopsies of the mammary gland and vagina at the beginning of treatment were compared with specimens obtained by biopsy or at autopsy at the conclusion of the experiments. Daily measurements of the vaginal desquamation were made according

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[1] These animals received the synthetic progesterone, Lutocylin, Ciba. It is a pleasure to acknowledge our indebtedness to the Ciba Pharmaceutical Products, Inc., Summit, N. J. for a generous supply of various hormones over a period of years, and Dr. E. Oppenheimer of their research department for his continued interest in our experiments and his many helpful suggestions.

[2] This animal received Prolution, Schering. For this and other generous gifts we take this opportunity of thanking the Schering Corporation, Bloomfield, N. J., through their research chemist, Dr. Erwin Schwenk, from whose wide knowledge we have profited on numerous occasions.

to the method previously described (1) as well as frequent recordings of the intensity of the color of the sex skin, which was graded by a color scale which ranged from 0 to 10, 8 being maximal for many normal females. *No.* 673 began to bleed vaginally 5 days after the last injection, was killed with gas on the same day, and received a vascular injection through the abdominal aorta with India ink which filled the larger endometrial arteries and veins, and entered an occasional spiral artery. *No.* 145C died of unknown cause during the course of the hormonal injections. This female had been used in numerous other experiments since castration but had received no hormonal treatment during the preceding year.

Observations

Sex skin. Following removal of the ovaries the sex skin of the monkey gradually fades from the varying shades of red that characterize its sexual maturity to a dull pallor that rarely exceeds 3 on our color scale. In the 2 animals that received progesterone in dosage of 20 mg. daily there was a definite increase in color intensity with a partial return of the brilliance of the sex skin. In *no.* 673 the color returned only to 5, whereas in *no.* 145C the response was more striking. Increased coloration was noted at the end of the first week and the intensity increased progressively, so that by the 25th day the sex skin had reddened to 8 from an initial intensity of less than 3. In *no.* 623, however, which received only 5 mg. of progesterone daily, no effect was noted on the sex skin coloration, which never exceeded 3. The daily threshold dose of progesterone for sex skin stimulation thus appears to lie between 5 and 20 mg. Unfortunately observations of the sex skin were not made during the course of injections of *no.* 673 at a dosage level of 10 mg.

Vagina. In common with all the other portions of the generative tract, the vagina undergoes pronounced atrophy after castration. The mucosa consists of a thin sheet of epithelial cells only a few layers deep, and in some places is completely denuded of epithelium. There is no differentiation of the layers and no cornification. Papillae or epithelial bulbs are absent, as are mitoses. The stroma is quite avascular and harbors subepithelial foci of round cell infiltration. Figure 1 depicts such a vaginal biopsy specimen from *no.* 145C which had been castrated for 4 years and had received no hormonal treatment for a year.

Under treatment with progesterone the epithelial cells proliferated and the number of layers increased several fold. The dark staining germinative layer became more sharply differentiated from the underlying submucosa, an effect already seen in *no.* 623 after 10 days of treatment. As *no.* 145C had received colchicine some hours before she died, many mitotic figures were to be seen in the growing epithelial papillae which become numerous and branched. Some of these changes are shown in figure 2 which represents a biopsy specimen from this animal 20 days after treatment was begun. Similar changes were apparent in the autopsy specimens recovered a week later. But not all of the vagina was thus "cured" of castration atrophy, for areas remained where round cells had collected in the stroma and others where the epithelium was but little proliferated.

At the beginning of the experiment there was practically no sediment in the vaginal lavage of all the animals, a condition typical of the castrate. It was difficult to find cells for study or to make a good vaginal smear. The lavage of *no.* 623, which received but 5 mg. per day of progesterone, remained clear throughout the period of treatment, which was also true of *no.* 673 when she received only 10 mg. per day of progesterone. When the latter received 20 mg. per day the percentage of sediment in the lavage remained at about 5 for a few days only soon after injections began, dropping to near 0 for the remainder of the period of treatment. Similarly, *no.* 145C

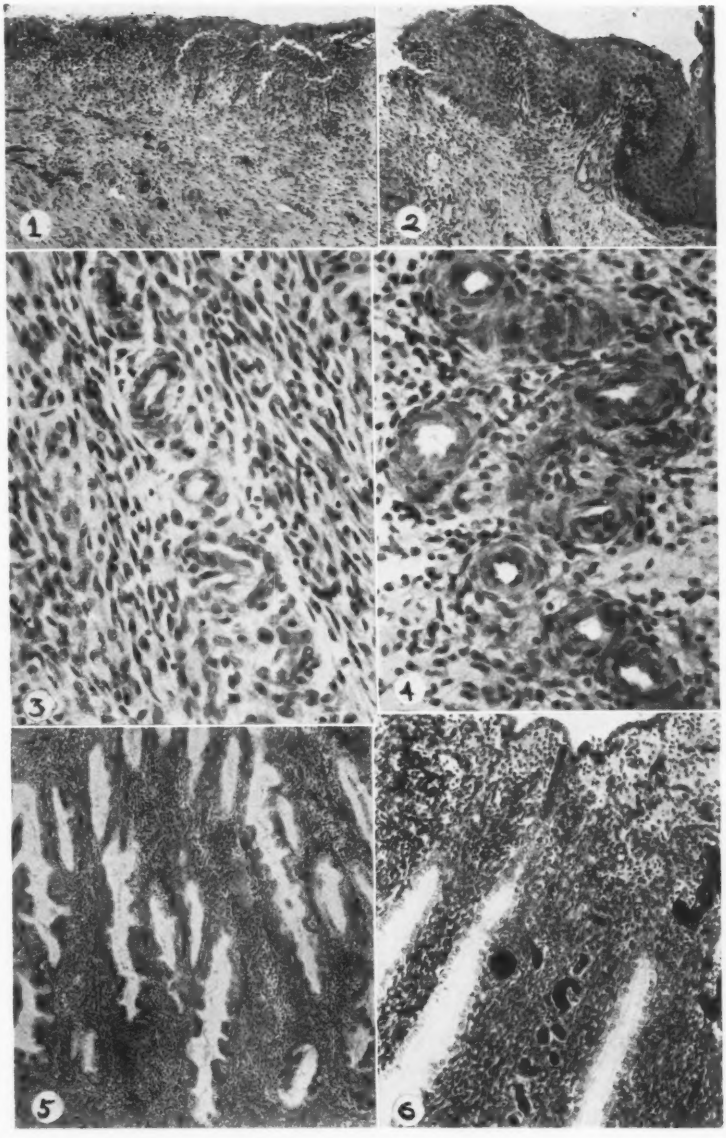

Fig. 1. Section of vaginal biopsy specimen of *no. 145C*, showing castration atrophy. ×78.

Fig. 2. Section of vaginal biopsy specimen of *no. 145C* after 27 days' treatment with 20 mg. daily of progesterone. ×78.

Fig. 3. Spiral artery from the endometrium of *no. 145C*, progesterone treated. ×230.

Fig. 4. Typical spiral artery from the normal endometrium of *no. 684*, removed 12 days after ovulation. ×200.

Fig. 5. Basal glands of endometrium of *no. 145C*, detail of figure 7. ×46.

Fig. 6. Section of uterus of *no. 673* on first day of bleeding after progesterone withdrawal. On the right is seen an injected spiral artery. ×115.

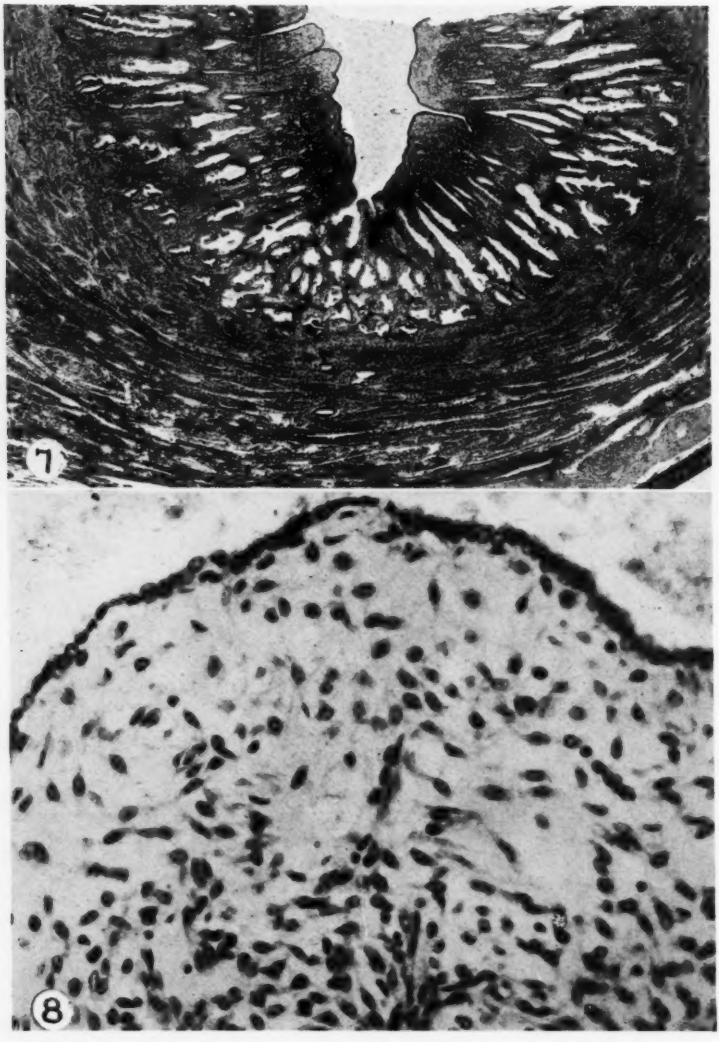

Fig. 7. Section of uterus of *no.* 145C after progesterone treatment. ×15.
Fig. 8. Same as figure 7, showing surface epithelium and stroma. ×300.

showed 5% desquamation from Feb. 6 to 17, a 'trace' the remainder of the period of treatment.

The amount of vaginal desquamation was, therefore, small as compared with that in estrogen-treated females.

Vaginal smears were made of *no.* 145C by the method of Papanicolaou (2) as modified by Shorr (3) from the 13th day of injection (2/12/41) to the time of death of the animal. The preparations are of interest, since by the 15th day of treatment a

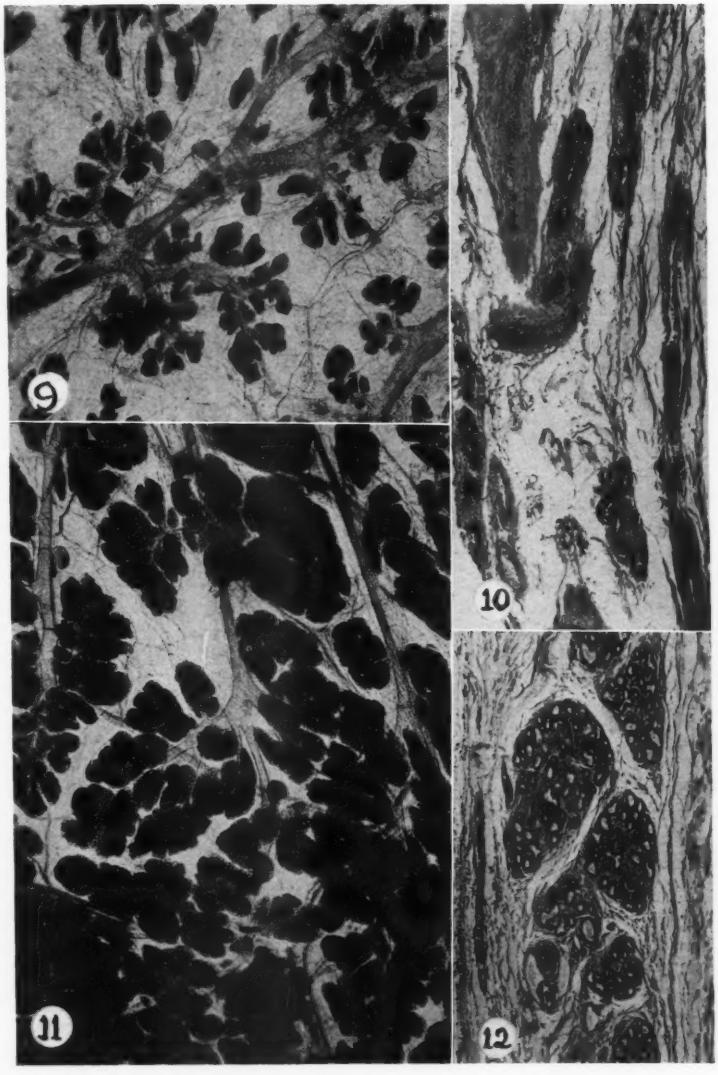

Fig. 9. Biopsy specimen of mammary gland of *no.* 673, showing castration atrophy. Whole mount; alum cochineal. ×12.

Fig. 10. Same as figure 9. Section. ×50.

Fig. 11. Mammary gland biopsy specimen *no.* 673 after 32 days' treatment with 20 mg. daily of progesterone. Whole mount; alum cochineal. ×12.

Fig. 12. Same as figure 11. Section. ×50.

considerable number of cornified cells were found to be present, at least cells which by their affinity for the red stain are interpreted as cornified cells. They are, of course, cells that are present at the peak of the estrogen effect both in the normal cycle

and during estrogen therapy. These increased in number until 2/25/41, when numerous epithelial or basal ('deep') cells appeared (usually found at the end of the normal menstrual cycle), as though the vaginal mucosa had become "fatigued" or less responsive after 30 days of treatment. Leukocytes were always present in the lavage in great numbers, which increased rather than decreased as the treatment went on. Sections of the cervix and vagina showed that the leukocytes were coming chiefly through the cervical epithelium.

Cervix. No estrogenic effect was apparent in sections of the cervix of the two animals that came to autopsy. This organ was lined with extremely thin cuboidal epithelium, so thin that it appeared to consist of little more than chains of contiguous nuclei. One had the impression that the cervical mucosa was "hyperinvoluted," for so thin an epithelium is never seen during the normal cycle, in contrast to the presence of numerous dividing cells in both vagina and uterus. This cervical atrophy was especially striking in *no.* 673 (1/9/41) and in addition masses of polymorphonuclear leukocytes filled the cervical lumen and infiltrated the submocosa where they were almost as numerous as the stromal cells themselves. In *no.* 145C some leukocytes were seen to pass through the thinner portion of the vaginal epithelium also and here the subepithelial infiltration of leukocytes was more marked than it was in the cervix.

The reaction of the cervix, then, under the influence of progesterone, offers a strong contrast to that seen after estrogen treatment which brings about greatly elongated cells ('picket fence' arrangement) which are highly secretory.

Uterus. Vaginal bleeding simulating menstruation in all respects occurred in *no.* 623 on the 3rd day after the cessation of injections, and in *no.* 673 bleeding had begun by the 4th day after the first course of injections. Following the second course of injections of *no.* 673, bleeding set in on the 5th day, at which time the animal was killed and perfused with India ink (fig. 6). *No.* 145C died while still under treatment and therefore before opportunity was afforded for withdrawal bleeding to occur. In three cases, therefore, withdrawal bleeding did occur, and in one of those which came to autopsy it was possible to study the uterus in sections to determine the source of the blood.

The uteri of *no.* 145C and *no.* 673, when examined at autopsy, gave definite evidence of growth resulting from progesterone. Bimanual palpation of *no.* 145C at the beginning of treatment revealed an infantile uterus. At autopsy the organ measured 17×14 mm. The uterus of *no.* 673 measured 18×12 mm. at autopsy, but palpation was not performed at the onset of treatment. The average measurements of the uteri of 6 macaques which had been castrated for 14 to 230 days were 11.4×7.7 mm., as reported by E. Allen (4), and the largest uterus in the series measured 12.5×8 mm. The uteri of our long-time castrates were even smaller. Comparison of these with the uteri of the progesterone treated monkeys thus affords confirmatory evidence of the growth-promoting influence of progesterone upon the castrate uterus.

The histologic picture of the endometrium of *no.* 145C disclosed a well proliferated organ the essential features of which were progestational in nature, that is, typical progesterone effects. The surface epithelium was low cuboidal (fig. 8). Beneath this the stroma was marked by edema fluid, and beneath this region the stroma was fairly loose. The stromal cells contained mostly large vesicular nuclei and well-marked, granular cytoplasm. The superficial glands were relatively straight and narrow but merged into the tortuous somewhat dilated glands of the basal layers (fig. 5). Here the glands presented the characteristic 'corkscrew' appearance of the premenstrual phase, with fraying of the cell borders and secretion in the lumen.

The uterus of *no.* 673 showed even more marked progestational changes in the

glands. In addition, the superficial layer of the endometrium presented the characteristic appearance of early menstruation, with heavy leukocytic infiltration of the stroma, subepithelial hematomata and free blood in the cavity (fig. 6).

In *no.* 145C the tissues of the endometrium teemed with mitotic figures in the stroma, the surface epithelium and the glands, except in the basal zone. At 5:00 P.M. on Feb. 25 an ointment containing 3 mg. of colchicine had been smeared on raw cotton and placed in the vagina of the animal (5). The next forenoon a vaginal biopsy was taken. The animal died in the early hours of the 27th, some 36 hours after the application of colchicine. It is possible that under these conditions the colchicine was still circulating in the blood stream in sufficient amounts to arrest mitoses in the uterus and exaggerate the real picture of mitotic activity.

The spiral arteries are of especial interest. In the India ink specimen (*no.* 673), basal arteries and veins were fully injected and enormous venous sinuses could be seen coursing over the functionalis. Only here and there was ink seen in the small arteries on their way toward the lumen (fig. 6). In both *no.* 673 and *no.* 145C the arteries (or arterioles which correspond to the spiral arteries of Daron) could with difficulty be found in thin sections, for they were weakly developed, lacking the connective tissue and muscle elements which make spiral arteries stand out boldly when viewed in sections of normal pregravid or gravid endometria. The contrast becomes apparent when one compares figure 3 taken from *no.* 145C with figure 4, recovered 12 days after ovulation. The latter normal specimen may be duplicated with preparations of uteri of castrates which had received estrogens, testosterone propionate or estrogens plus progesterone (cf. fig. 4, 12 and 16 of Rossman's study (6)). With all of these reagents one sees compact thick-walled spiral arteries distinctly set off from the surrounding stroma. Nevertheless, menstrual bleeding occurred in the 3 progesterone-treated animals which lived after hormone-withdrawal.

Mammary glands. Following castration a generalized atrophy of the macaque mammary gland occurs, in addition to the formation of localized areas of nodular hyperplasia (7). Atrophy is usually sufficiently advanced by one month after removal of the ovaries so that the gland may be used as an indicator of the mammatropic effects of test substances. Biopsies of the mammary glands at the beginning of treatment revealed the typical castrate pattern of small shrunken lobules with few collapsed acini (fig. 9, 10). The acinar cells were small and contained dark-staining pyknotic nuclei. Following treatment with progesterone a striking increase in the size and number of the lobules was noted (fig. 11, 12). The acini were greatly increased in number and the individual cells were larger, with increased amounts of cytoplasm and round vesicular nuclei. Numerous mitotic figures were seen. These changes were most marked in *no.* 673 after 32 days of treatment with 20 mg. of progesterone daily. Similar but less striking growth effects were observed in *no.* 623 and *no.* 145C.

DISCUSSION

It is interesting to compare the effects of progesterone on the secondary sex organs of the monkey with the changes produced by similar doses of desoxycorticosterone, a steroid of close chemical relationship. Both substances produced reddening of the sex skin and mammary development, and both caused withdrawal bleeding from castrate uteri which had not been previously primed with estrogens (8). However, while increased vaginal desquamation resulted from the administration of desoxycorticosterone, progesterone had no such effect.

Hisaw, Greep and Fevold (9) have shown that when progestin is administered to adolescent macaques the edema and redness of the sex skin produced by previously injected estrogens are modified and the effects of concurrently injected estrogens

inhibited. Marker and Hartman (10) have observed a similar inhibitory effect of a huge dose of progesterone on the reddening of the sex skin in a pregnant monkey. In the present experiments also, the sex skin remained pale while subjected to a daily progesterone dosage of 5 mg. When the dosage of progesterone was increased to 20 mg. per day, however, definite reddening of the sex skin occurred. We have no explanation to offer for this phenomenon. It is, however, only one of the surprises encountered in this laboratory relative to the coloration of the sex skin of the monkey. Thus, for example, the pregnant and also the lactating rhesus monkey maintain almost maximal reddening of the buttocks in spite of complete castration early in pregnancy (11). There is some reason for expecting the pregnant female to show the phenomenon, for the internal secretion of the placenta may be involved in explanation; but that the lactating female should maintain the redness, heretofore considered an estrogenic effect, requires further search for the cause. It is possible that in the present experiments progesterone is partially transformed into a substance that affects the sex skin but is not itself antagonized by progesterone. It is more likely, however, that there is another source of the factor which affects the blood supply of the skin, possibly the adrenals. In view of the fact observed by one of us (C.G.H.) that anterior pituitary extracts concurrently administered with estrogens, heightens the sex skin effect of the latter, it is inviting to bring the hypophysis into the picture. Thus the anterior pituitary might be stimulated to produce adrenotropic hormone (resulting in the output of the appropriate steroids) by the injected progesterone, by the chorionic hormones in pregnancy, by nerve impulses coming from the suckled breasts.

The control of the blood supply of the sex skin is not as simple as has heretofore been supposed. It should be pointed out, in passing, that this problem is not of mere academic interest, for it is well known that sex hormones affect the dermal blood supply in men and women, hence the physiology of its control is of importance to the internist and especially the gynecologist. It is suggested that the sex skin of the monkey offers the best test object for studying the problem.

When we speak of a reddening of the sex skin in the monkey as an estrogenic effect we are speaking historically, since estrogen was the first hormone studied. It is true that the estrogens are effective in a hundred-fold lower dosage, by weight, than the other steroids. Nevertheless, the action of the estrogens on the sex skin can hardly be said to be specific when identical effects may be attained with testosterone (12), desoxycorticosterone (8) and progesterone.

Progestin has been previously found to have no effect on the castrate vagina when administered without estrogen. W. Allen and Meyer (13) have reported dosages of progestin up to 4 RB.U. in 8 days ineffectual in mice, and Hisaw, Greep and Fevold (14) noted a similar failure of the monkey vagina to respond to 4 RB.U. of progestin daily for 10 days, although the latter authors (9) subsequently reported slight vaginal stimulation by a similar dosage of progesterone. Definite epithelial growth as well as cornification was observed in the present experiment as a result of the daily administration of 10 and 20 mg. of progesterone, but the number of leukocytes in the vaginal smear increased rather than decreased. This, however, is due to the effect of progesterone on the cervix, the source of the leukocytes, for this organ shows involutionary changes and not growth responses.

Hormonal extracts capable of producing progestational proliferation in the uterus of the rabbit were first prepared by Herrmann (15) from corpora lutea, although it is doubtful whether his extracts actually contained any progestin. It was not until 1930 that premenstrual changes were experimentally produced in the uterus of the castrate monkey, through the combined use of estrogen and crude luteal hormones

(16). In 1936 Hisaw and Greep (17) obtained a similar premenstrual endometrium with a combination of estrogen and progesterone. It had previously been shown in the castrate rabbit that extracts of sow corpora lutea were unable to modify the uterus when given without estrogens or estrogen priming (18). W. Allen (19) subsequently found that complete progestational proliferation could be produced in a long-time castrated rabbit by estrogen-free progestin and in a 20-day castrate by pure progesterone. These results were irregular, however, and Allen has repeatedly emphasized the importance of preliminary and concomitant estrogenic stimulation for the full effect of progesterone on the structure of the uterus.

More recent observations have shown that when larger dosages of progesterone are used, similar histologic changes can be uniformly attained in the castrate uterus without the aid of estrogen. Thus Selye (20) has produced progestational uteri in rats with 10 to 15 mg. of progesterone daily for 10 to 20 days. His animals had been castrated for periods only up to 9 days, however. Using adult mice which had been castrated for various intervals up to 70 days, Hooker (21, 22) has found 0.25 to 1 mg. of progesterone daily capable of inducing mitoses in all the tunics of the uterus and of conditioning the formation of traumatic deciduomata. The only previously successful production of premenstrual changes in primates by progesterone alone was reported by Hisaw, Greep and Fevold (14) in a young adult monkey which had been castrated for 242 days and was treated with 4 mg. of progesterone daily for 18 days.

The importance of the dosage factor in determining the response of the castrate uterus to progesterone is demonstrated also by the occurrence of withdrawal bleeding. Zuckerman (23) treated a spayed monkey with 2 RB.U. of progestin (equivalent to 2 mg. of progesterone) daily for 14 days. Uterine bleeding failed to occur either during or following this course of injections. Dosages of progesterone up to 5 mg. daily for 14 days have likewise failed to cause bleeding after cessation of treatment (24). The present experiments represent the first successful induction of uterine bleeding in castrated monkeys by progesterone without pretreatment with estrogen. A 5 mg. daily dosage (for 32 days, in contrast to the 14 days in Engle's experiment) was as effective as the larger dosages of 10 and 20 mg. in inducing withdrawal bleeding.

Of more than passing interest is the finding here reported that menstruation occurred in typical fashion in the presence of atypical spiral arteries. The intense spiralling and well developed tunics which one sees in normal pregravid or early gravid uteri seem, therefore, to be unessential prerequisites to menstrual bleeding. Whatever changes in the spiral arteries are necessary for that still poorly understood phenomenon, the menstrual bleeding, the prerequisites may be physiological rather than anatomical peculiarities, confined to the primates.

Numerous reports have attested to the inadequacy of progestin alone in producing mammary proliferation or lobule development in castrated rabbits (25, 26, 27), rats (28), and male guinea pigs (29). Experiments with progesterone have likewise shown it to be without effect on the mammary gland of the castrated rabbit (30). Astwood and Geschickter (31) found no demonstrable effect on the rat mammary gland from 1 mg. of progesterone daily for 20 days or 2 mg. daily for 8 days. Failure of the rat breast to respond to 4 mg. of the hormone daily was likewise noted by Selye, Browne and Collip (32). The more recent experiments of Selye (20, 33) have clearly indicated, however, that when recently castrated rats were treated with dosages of progesterone up to 15 mg. daily marked mammary development and lobule formation occurred. Yet even with this large dosage, when the institution of treatment was delayed until 9 days after castration, only slight development of the gland was pro-

duced. The present experiments, on the other hand, clearly demonstrate that even as long as 4 years after castration and 1 year after exogenous hormonal stimulation, the mammary gland of the monkey responds by definite lobular development to treatment with 20 mg. of progesterone daily.

SUMMARY

Stimulation of the secondary sex organs of castrated rhesus monkeys was produced by treatment with 5 to 20 mg. of progesterone daily for periods ranging from 20 to 32 days.

Reddening of the sex skin was produced with a 20-mg. daily dosage, whereas 5 mg. daily was ineffectual.

Growth and cornification of the vaginal mucosa occurred, but there was no consistent increase in the vaginal desquamation.

Growth of the uterus occurred, with progestational proliferation of the endometrium. Following cessation of treatment, characteristic menstrual bleeding occurred within 3 to 5 days.

Development of the mammary gland, with lobular proliferation of the acini, was demonstrated.

Although progesterone normally acts in the body in conjunction with estrogens and on organs which have received preliminary estrogenic priming, with adequate dosages of progesterone alone the characteristic reactions of this hormone may be evoked, even after long periods of castration atrophy.

The specificity of action of the steroidal hormones is less strict than has heretofore been supposed.

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NOTES AND COMMENTS

THE PRODUCTION OF GLYCOSURIA IN THE NORMAL RAT BY MEANS OF 17-HYDROXY-11-DEHYDROCORTICOSTERONE

THE DIABETIC STATE OF THE PARTIALLY DEPANCREATIZED RAT can be intensified by the administration of anterior pituitary extract,[1] certain of the adrenal steroids[2,3] and by estrogenic substances[4,5]. Although a diabetic state can be induced in the normal dog by means of anterior pituitary extracts, the normal rat has not yet been shown to develop glycosuria when treated with this principle. A mild glycosuria and a hyperglycemia can be induced in normal rats by the administration of stilbestrol[5]. The present report concerns the production of severe glycosuria and hyperglycemia in 3 normal rats by the administration of one of the steroid compounds obtained from the adrenal cortex, 17-hydroxy-11-dehydrocorticosterone.

Male rats of the Sprague-Dawley strain were used in these experiments. The animals were maintained on a diet of Purina Dog Chow until they reached a weight of approximately 300 gm. They were then placed in metabolism cages and fed a high carbohydrate fluid diet composed of the following constituents:

starch	540 gm.
dextrin	260 gm.
sucrose	265 gm.
egg albumin (Merck)	200 gm.
Osborne, Mendel salt mixture	40 gm.
Cellu-flour	120 gm.
Vi-Penta (Roche)	10 cc.
wheat germ oil	10 cc.
Mazola oil	10 cc.
water to make total volume	2000 cc.

The nitrogen content of this diet was found by analysis to be 1.66 gm. per 100 cc.

The diet was administered by stomach tube each morning and evening, the total daily amount being 26 cc. It is necessary to begin administering the diet in small amounts, but after the 4th day the full feeding can be administered. During the first week of administering a high carbohydrate diet by stomach tube small amounts of glucose are excreted, but tolerance to the diet develops rapidly so that spontaneous glycosuria never reappears, and such rats can be satisfactorily maintained in this way for indefinite periods of time.

The 17-hydroxy-11-dehydrocorticosterone was administered in sesame oil by subcutaneous injection twice daily. Twenty-four-hour specimens of urine were collected and preserved with toluene. The methods used for analyses have been described by Evans [6].

Three normal rats were studied. During the first few days of the control period they excreted small amounts of glucose. All rats became adapted to the diet so that the urine was free of glucose for at least 10 consecutive days before treatment with 17-hydroxy-11-dehydrocorticosterone was begun. *Rat 120* received 1 mg. per day for 7 days, 2 mg. per day for 7

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days, and 5 mg. per day for 10 days. *Rat 401*, was treated with 5 mg. per day for 10 days. *Rat 412* was treated with 10 mg. per day but succumbed during the 4th day of treatment.

RESULTS

Body weight. As recorded in figures 1 and 2, all 3 rats gained in weight during the control

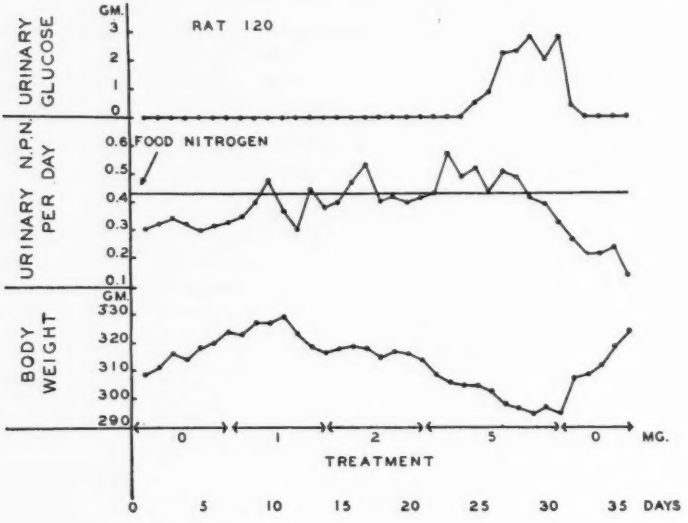

Fig. 1. Glycosuria in the normal rat during treatment with 17-hydroxy-11-dehydrocorticosterone.

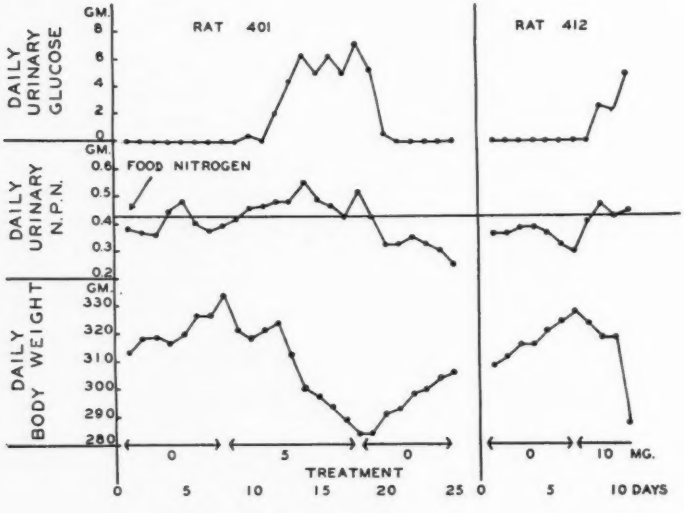

Fig. 2. Glycosuria in 2 normal rats during treatment with 17-hydroxy-11-dehydrocorticosterone.

period and lost weight during the administration of the hormone. *Rats 120* and *401* which survived gained in weight after the administration of the hormone was stopped. *Rat 412* appeared to be markedly dehydrated at the time of death.

Urinary excretion of glucose and nitrogen. In *rat 120* the excretion of non-protein nitrogen was increased above the control values during the administration of 1 and 2 mg. daily of the hormone, but glycosuria did not develop until 5 mg. daily were administered. For 6 consecutive days the animal excreted more nitrogen than was contained in the food. When the administration of the hormone was stopped the glycosuria disappeared and the nitrogen balance became distinctly positive.

In *rat 401*, the administration of 5 mg. daily of the hormone caused the development of severe glycosuria with a negative nitrogen balance. When the administration of the hormone was stopped the glycosuria ceased and nitrogen retention occurred.

In *rat 412*, the administration of 10 mg. of the hormone daily caused the development of glycosuria and a negative nitrogen balance. The rat succumbed during treatment.

All animals were free of acetonuria throughout the experiments.

Blood sugar. The blood sugar level was studied in *rats 120* and *401*. During the control period the values at 4 consecutive two-hour intervals after feeding were elevated only a little above those of the fasting level. During the administration of 5 mg. of the hormone daily to each rat the values for blood sugar were elevated even during fasting. These data are summarized in table 1.

TABLE 1. BLOOD SUGAR VALUES IN NORMAL RATS EXPRESSED IN MG.%. The control values were obtained on the day prior to beginning the administration of the hormone. The values for the injection period were obtained on the 6th day that the 5 mg. daily dose of 17-hydroxy-11-dehydrocorticosterone was given.

Rat No.	Phase of experiment	16 Hr. fast	Hours following feeding			
			2	4	6	8
120	Control	82	124	118	98	90
	Injection	139	268	304	292	218
401	Control	91	93	91	83	86
	Injection	242	346	334	305	295

Organ weights. The adrenals, thymus and testes of *rats 120* and *401* were weighed at necropsy 7 days after stopping the administration of the hormone. These data, together with the range of values in untreated rats of similar weight are given in table 2. The gonads and thymus of each of the 2 rats were atrophic.

TABLE 2. A COMPARISON OF ORGAN WEIGHTS IN RATS TREATED WITH 17-HYDROXY-11-DEHYDROCORTICOSTERONE WITH THE RANGE OF VALUES IN 10 UNTREATED RATS

Animals	Adrenal	Thymus	Testes
	mg.	*mg.*	*mg.*
Normal	32–46	185–419	2748–3538
Rat 120	57	38	575
Rat 401	27	36	1057

DISCUSSION

As far as the author is aware this is the first production of a prolonged hyperglycemia and glycosuria in the normal rat by means of any substance isolated from animal tissues. The temporary diabetes induced in these animals is clearly different from the short periods of hyperglycemia and glycosuria resulting from glycogenolysis as produced by epinephrine and similar agents. A mild glycosuria which is superficially similar to the diabetic state described here has been produced in the normal force-fed rat by means of stilbestrol.

Not all of the glucose excreted under the influence of 17-hydroxy-11-dehydrocorticosterone could be accounted for on the basis of increased carbohydrate formation from protein.

Ingle and Thorn[7] observed similar results in the partially depancreatized rat treated with this hormone. It is probably necessary to assume that the hormone inhibits the utilization of carbohydrate or alters the interconversion of the foodstuffs in addition to its effect in stimulating the catabolism of protein[2].

The death of *rat 412* is believed to have been caused by the hormone. The relationship of simple toxicity and of the diabetic state in causing death is not known. It has frequently been observed in this laboratory that when the force-fed, partially depancreatized rat is stimulated with a diabetogenic agent so that the diabetes is precipitated rapidly the animal may promptly lose excessive amounts of water, develop coma and die. The state of shock can be reversed by treatment with insulin. Death may occur before the rat becomes totally diabetic and without the excretion of ketones in the urine. The syndrome requires further study.

It has been found by Dohan and Ingle[8] that after adjustment the normal rat can tolerate far larger amounts of carbohydrate than were fed in these experiments without the excretion of any glucose in the urine. Moreover, when amounts of pancreas up to 90% of the total are removed these animals still retain the capacity to adapt to force-feeding so that the amount of carbohydrate administered in these experiments is tolerated without the excretion of any glucose in the urine. These considerations lend further emphasis to the fact that 17-hydroxy-11-dehydrocorticosterone has a marked effect on the carbohydrate metabolism of normal rats.

Because of the successful production of permanent diabetes in the normal dog by means of anterior pituitary extract[9] the possibility of producing permanent diabetes in the rat by prolonged treatment with adrenal steroids has been considered, but the limited supply of this compound prevents undertaking the experiment.

SUMMARY

The administration of 17-hydroxy-11-dehydrocorticosterone to normal rats which were force-fed with a high carbohydrate diet caused hyperglycemia, glycosuria, an increased excretion of non-protein nitrogen, a marked loss in weight, and testis and thymus atrophy.

I wish to express my appreciation to Dr. J. J. Pfiffner of Parke, Davis and Co., Detroit, Michigan, who supplied the 17-hydroxy-11-dehydrocorticosterone.

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[7] INGLE, D. J., AND G. W. THORN: *Am. J. Physiol.* 132: 670. 1941.
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[9] YOUNG, F. G.: *Endocrinology* 26: 345. 1940.

THYROID HORMONE IN BLOOD. II.

THE AUTHORS[1] SHOWED THAT POTASSIUM IODIDE was completely extracted from finely divided blood by 20 volumes of methanol followed by 20 volumes of acetone, but that thyroglobulin was not extracted. Therefore the iodine left in the blood after this double extraction was referred to as "hormone iodine." The senior author[2] showed that the values obtained by this method were the same as those obtained by ultrafiltration or by dialysis for 5 days. Because of the danger of leakage of an ultrafilter or dialyzer and the time consumed, these methods were discontinued.

McClendon and Foster when determining 'hormone iodine' in 373 patients found that many had taken various iodine drugs. They advocated prolonging the methanol extraction for one hour in a shaking or rotating machine in an attempt to remove these drugs[3]. Even one hour of extraction failed to remove iodide of mercury.

Salter[4] criticizes the methods in the literature and advocates precipitation of the thyroid iodine in the blood with acetic acid or with zinc sulfate and sodium hydroxide. Since much of the speculation on the subject concerns the solubility of thyroxine, it seemed worthwhile to make an attempt to determine the solubility of thyroxine in some solvents that might be used in extracting potassium iodide from blood.

Experimental: An excess of synthetic thyroxine was added to each of six 100 cc. glass-stoppered Erlenmeyer flasks. Each of these was filled with a different solvent and shaken for an hour, and then left in a thermostat box at 25°C. for 1 month. At the end of that time aliquots of the solvent were analyzed for iodine. The flasks were then shaken, placed in a thermostat box at 30°C. for 3 months and aliquots analyzed at the end of the time with the results that are given in table 1.

TABLE 1. SOLUBILITY OF THYROXINE[1]

Solvent	Micrograms iodine per cc.	
	at 25° C.	at 30° C.
water	0.1	0.1
methanol	8.7	13.3
ethanol	4.9	5.8
i-propanol	0.6	0.6
i-butanol	1.4	1.9
acetone	3.4	5.7

[1] Hoffman, La Roche, Nutley, N. J.

It may be seen that thyroxine shows a maximum solubility in methanol. Its solubility in acetone is almost as great as in ethanol, therefore if it is desired to retain free thyroxine in blood the solvents must be carefully chosen. From reports by Kendall[5] one is led to believe that the sodium salt of thyroxine is more soluble in alcohols than is the free amino acid. But it has not been shown that blood contains free thyroxine or its sodium salt. Kendall found 8 times as much thyroxine in water after precipitation with CO_2 as we were able to dissolve. Our solution may have been undersaturated and Kendall's supersaturated.

Trevorrow[6] believes all of the iodine compounds were extracted from the blood with alcohol in 28 hours or with acetone in 12 hours, or in 1 hour from small quantities of blood. The data in table 1 in her paper indicate that 2.7 micrograms of iodine were extracted in 28 hours with alcohol from 50 cc. dog blood, 2.5 micrograms from 50 cc. human blood and 2.6 micrograms from 50 cc. beef blood. It is the opinion of many blood-iodine-microchemists that

[1] MCCLENDON, J. F., AND W. C. FOSTER: *Proc. Soc. Exp. Biol. & Med.* 39: 230. 1938.
[2] MCCLENDON, J. F.: Iodine and Incidence of Goiter. U. of Minn. Press, 1939.
[3] MCCLENDON, J. F., AND W. C. FOSTER: *Endocrinology* 28: 412. 1941.
[4] SALTER, W. T.: The Endocrine Function of Iodine. Harvard Univ. Press. 1940.
[5] KENDALL, E. C.: Thyroxine. Am. Chem. Soc. Monograph. 1929.
[6] TREVORROW, V.: *J. Biol. Chem.* 127: 737. 1939.

50 cc. of blood should contain about 4 or 5 micrograms of iodine. Table 2 in the same paper indicates that with acetone 17.2 micrograms of iodine were extracted from 600 cc. of beef blood in 12 hours. By the same criterion this would be expected to contain 50–60 micrograms of iodine. The residue was not analyzed.

We took a sample of human blood and in 5 cc. found 0.22 microgram of thyroid hormone iodine by our 1 hr. extraction method. Five cc. were extracted in a Soxhlet apparatus 28 hours with 3 portions of methanol and 0.2 microgram of iodine was found in the residue. Obtaining another sample of human blood we found 0.24 microgram of thyroid hormone iodine in 5 cc. by our 1-hour extraction method, and in another 5 cc., after extraction for 16 hours in a Soxhlet apparatus with acetone, we found 0.22 microgram iodine in the residue.

One objection to dialysis for removing potassium iodide from blood is the danger of leakage, but if leakage can be prevented it is convenient since the sample is packed in a ⅜ inch dialyzer tube for iodine analysis. Heat coagulation was attempted to insure against leakage. Five cc. of blood were pipetted into the dialyzer tubing as in the McClendon and Bratton[7] method for total iodine. The tube was tied at each end and the strings tied to a rectangular frame 3″ ×6″ (made of a glass rod) so that the tube was stretched. The frame was plunged into a 1 liter pyrex beaker filled with boiling distilled water and set aside to dialyze overnight. The frame was then suspended 3″ from a 250-watt Westinghouse reflector drying lamp for 2 hours, then the tube was cut off and the contents analyzed for iodine by the McClendon-Bratton method.

Fifteen cc. samples of oxalated human blood were subjected to analysis by the 3 methods, (*a*) the original double extraction (methanol-acetone) method of McClendon and Foster; (*b*), the same with 1 hour agitation of the methanol and (*c*), the overnight dialysis technic described above. The results are given in table 2.

TABLE 2

Blood sample	Micrograms 'hormone iodine' per 5 cc. blood		
	methanol acetone	methanol agitated 1 hr. acetone	dialyzed 12 hrs. after heat coag.
1	.32	.23	.35
2	.37	.29	.37
3	.30	.23	.28
4	.29	.28	.37
5	.47	.43	.40
6	.36	.30	.35
7	.38	.34	.39

Conclusions. At least a large part of the thyroid hormone in the blood is non-dialyzable, is insoluble in methanol or acetone and is not free thyroxine, because 1 cc. of methanol or acetone or 5 cc. of water would dissolve more free-thyroxine-iodine than the total 'thyroid hormone iodine' in 5 cc. of blood. Reports that thyroxine added to blood is not extracted by alcohol or acetone (or only with extreme slowness) may be due to physical or chemical union with, or occlusion by blood proteins. We have extracted more than 400 samples of blood with alcohol or acetone or both and have never extracted all of the iodine compounds even after 28 hours of continuous extraction.

Summary. All of the blood iodine cannot be extracted by methanol in 28 hours or by acetone in 16 hours in a Soxhlet extractor.

The values for thyroid hormone iodine obtained by the original method[1] or by 12 hours of dialysis herein described are, on the average, nearly 20% higher than those obtained after one hour of agitation in methanol.[3]

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[7] McCLENDON, J. F., AND A. C. BRATTON: *J. Biol. Chem.* 123: 699. 1938.